DOCTOR OF PHILOSOPHY

Investigating the cellular mechanisms that determine RNMT dependency in breast cancer cells

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Finally, I would like thank my mum and dad for their unconditional love and for all their encouragement throughout my academic studies.

This thesis is dedicated to my nana. I would not be where I am today without her love and guidance.

Declarations

I hereby declare that the following thesis is based on the results of investigations conducted by myself, and that this thesis is of my own composition. Work other than my own is clearly indicated in the text by reference to the researchers or their publications. This dissertation has not in whole, or in part, been previously presented for a higher degree.

……..

Shanade Dunn

I certify that Shanade Dunn has spent the equivalent of at least nine terms in research work in the College of Life Sciences, University of Dundee, and that she has fulfilled the conditions of the relevant Ordinance and Regulations of the University of Dundee and is qualified to submit the accompanying thesis in application for the degree of Doctor of Philosophy.

……..

Dr. Victoria Cowling

Abbreviations

˚C: degree Celsius

4E-BP: eIF4E-binding protein

ANOVA: analysis of variance

AMPK: AMP-activated protein kinase

BSA: bovine serum albumin

CBC: cap-binding complex

cDNA: complementary DNA

ChIP: chromatin immunoprecipitation

COSMIC: Catalog of Somatic Mutations In cancer

CPM: counts per minute

CTD: carboxyl-terminal domain

Da: dalton

DNA: deoxyribonucleic acid

DRB: 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole

DTT: dithiothreitol

EGF: epidermal growth factor

eIF: eukaryotic translation initiation factor

DEPC: diethylpyrocarbonate

DMEM: dulbecco’s modified eagle’s medium

DMSO: dimethyl sulphoxide

DSTT: Division of Signal Transduction Therapy

ECL: enhance chemiluminescence

FACS: fluorescence activated cell sorting

FBS: foetal bovine serum

EGTA: Ethylene glycol tetraacetic acid

EDTA: Ethylenediamine tetraacetic acid

ELISA: enzyme-linked immunosorbent assay

GFP: green florescent protein

GMP: guanosine monophosphate

GTP: guanosine 5’- triphosphate

HEK-293: human embryonic kidney-293

HER2: human epidermal growth factor receptor 2

Hr: hours

IMEC: immortalised mammary epithelial cells

IP: immunoprecipitation

IRES: internal ribosomal entry sites

kDa: kilodalton

LKB1: liver kinase b1

methyl cap: 7-methylguanosine cap

Min: minutes

mRNA: messenger RNA

mTOR: mammalian target of rapamycin

Myc: myc proto-oncogene protein

NTPs: nucleoside triphosphate

RAM: RNMT activating mini-protein

RNA: ribonucleic acid

RNA Pol II : RNA polymerase II

RNGTT: RNA guanylyltransferase and triphosphatase

RNMT: RNA guanine-7 methyltransferase

PAGE: polyacrylamide gel electrophoresis

PARP: poly (adenosine diphosphate [ADP]-ribose) polymerase

PBS: phosphate buffered saline

PCR: polymerase chain reaction

PDK1: 3- phosphoinositide dependent kinase 1

$PI(3,4,5)P_3$: phosphatidylinositol 3,4,5-triphosphate

$PI(4,5)P_2$: phosphatidylinositol 4,5, bisphosphate

PI3K: phosphoinositide 3-kinase

PIK3CA: phosphoinositide 3 kinase catalytic alpha

PTEBb: positive transcription elongation factor b

PTEN: phosphatase and tensin homolog

RNA: ribonucleic acid

RNAi: ribonucleic acid interference

rRNA: ribosomal RNA

rRNasin: recombinant RNasin ribonuclease inhibitor

RNGTT: capping enzyme

RNMT: RNA (guanine-7) methyltransferase

RNP: ribonucleoprotein

RTK: receptor tyrosine kinase

S6K: p70 ribosome S6 kinase

SAH: s-adenosyl homocysteine

SAHH: s-adenosyl homocysteine hydrolase

SAM: s-adenosyl methionine

SD: standard deviation

SDS: sodium dodecyl sulphate

SDS-PAGE: sodium dodecyl sulphate polyacrylamide gel electrophoresis

Ser: serine

SGK: serum and glucocorticoid regulated protein kinase

SH2: src homology 2

siRNA: small interfering RNA

snRNA: small nuclear RNA

STA: straurosporine

TFIIH: transcription factor II human

Thr: threonine

TLC: thin layer chromatography

TN: triple negative

TORKinibs: second generation mTOR kinase inhibitors

tRNA: transfer RNA

Tris: tris (hydroxymethyl) methylamine

TSC: tuberous sclerosis complex

TUNEL: terminal deoxynucleotidyl transferase nick end labelling

Tyr: tyrosine

UTR: untranslated region

WT: wild-type

Amino Acid Code

Amino acid	One letter symbol
Alanine	A
Arginine	R
Asparagine	N
Aspartate	D
Cysteine	C
Glutamate	E
Glutamine	Q
Glycine	G
Histidine	H
Isoleucine	I
Leucine	L
Lysine	K
Methionine	M
Phenylalanine	F
Proline	P
Serine	S
Threonine	T
Tryptophan	W
Tyrosine	Y
Valine	V
any amino acid	X

Abstract

Breast cancer is the leading cause of cancer death in women worldwide. Although significant advances have been made in the treatment of breast cancer, new therapeutic approaches are required. Protein synthesis is often found to be deregulated in cancer and there has been significant effort into developing strategies to inhibit protein synthesis for anti-cancer therapeutics. The addition of the 7-methylguanosine cap (methyl cap) at the 5' end of mRNA is essential for efficient protein synthesis and cell viability. In humans, RNA guanine-7 methyltransferase (RNMT) methylates the guanosine cap. RNMT expression is essential for cell viability and efficient gene expression.

This thesis demonstrates that a subset of breast cancer cells exhibit an enhanced dependence on RNMT for survival, in comparison to immortalised mammary epithelial cells. Moreover, RNMT depletion induces apoptosis in a subset of breast cancer cell lines. Further analyses reveal that this cellular sensitivity to RNMT depletion does not correlate with cellular RNMT expression, RNMT enzymatic activity, global protein synthesis levels, basal cell proliferation rate or c-Myc protein expression. It was found that activating mutations in the gene PIK3CA, which encodes the p110α catalytic subunit of PI3K, contributes to cellular RNMT dependency. Moreover, inhibition of PI3K signalling in a RNMT depletion sensitive breast cancer cell line reverses the sensitivity. These findings provide the first evidence that RNMT dependency in breast cancer is related to PI3K activity/ signalling. Since an estimated 18-40% of breast tumours have activating mutations in PIK3CA, my findings could potentially have significant therapeutic implications.

Chapter 1: Introduction

1.1 Gene expression

1.1.1 The gene expression pathway

Gene expression is the process by which genetic information is used to direct the production of protein. During gene expression, DNA is transcribed into RNA and then RNA is translated into protein. This multistep process was first described by Francis Crick and is known as the central dogma of molecular biology. Higher eukaryotes consist of many different cell types; each performing a specific function and displaying a unique pattern of gene expression. Every one of these cells contain the same genetic information and the remarkable cellular diversity is achieved through the differential regulation of genes. Cells respond to environmental and intracellular signalling by regulating the expression of their genes. Gene expression in higher eukaryotes is known to be regulated at multiple stages including: transcription, pre-mRNA processing (such as the addition of the 7-methylguanosine cap at the 5' end of mRNA), mRNA nuclear export and mRNA translation (Orphanides and Reinberg, 2002). The tight regulation of gene expression is required for normal cell proliferation. Consequently, deregulated gene expression can lead to abnormal cell proliferation and is often found to be associated with cancer (White, 2008, Blagden and Willis, 2011, Silvera et al., 2010).

Following transcription by RNA Polymerase (Pol) II, pre-mRNAs undergo a series of processing events which result in the production of mature translatable mRNA. The addition of the 7-methylguanosine cap (methyl cap) at the 5' end of mRNA is the first pre-mRNA processing event to occur. The methyl cap is

known to be essential for efficient gene expression and cell viability. This thesis focuses on investigating whether targeting the methyl cap is a viable strategy for inhibiting breast cancer cell proliferation.

This thesis introduction describes the formation and function of the 7-methylguanosine cap and the deregulation of gene expression in cancer. This introduction also describes the development of cancer and particularly focuses on breast cancer, which is of special relevancy for this thesis. However, the basic processes of the gene expression pathway will first be described in this subchapter.

1.1.2 Transcription initiation

The first step in gene expression is the transcription of DNA into RNA; a process which occurs in the nucleus. RNA Pol II is the multi-subunit enzyme which is responsible for the transcription of mRNAs, some micro RNAs and some small nuclear RNAs (snRNAs). The formation of a transcription competent RNA Pol II complex at the gene promoter is essential for the initiation of transcription. The large subunit of RNA Pol II contains a carboxyl-terminal domain (CTD) which consists of heptad repeats of the sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser (52 repeats in mammals) and it is essential for RNA Pol II function (Hsin and Manley, 2012, Bartolomei et al., 1988). Several residues of the CTD heptad repeats are dynamically phosphorylated during transcription, with serine 2 and 5 being the best characterised. Serine 5 phosphorylation is mediated by the kinase subunit of Transcription Factor II Human (TFIIH) and is primarily detected in gene promoters (Trigon et al., 1998, Komarnitsky et al., 2000b). Serine 2 CTD phosphorylation is mediated by the kinase subunit of Positive Transcription Elongation Factor b complex (PTEFb)

and it is found predominately in gene bodies (Marshall et al., 1996, Komarnitsky et al., 2000b). Following transcription initiation, the CTD is phosphorylated on serine 5 and RNA Pol II subsequently pauses at promoter proximal sites. RNA Pol II pausing is known to be an important event in the control of transcription (Saunders et al., 2006, Adelman and Lis, 2012). The CTD phosphorylation of serine 2 then stimulates RNA Pol II to release from pausing, thereby allowing transcription elongation to proceed (Egloff and Murphy, 2008, Zhou et al., 2012). The phosphorylated CTD serves as a binding platform for several pre-mRNA processing proteins, including the enzymes which form the mRNA methyl cap, thereby recruiting these proteins to the transcript (Komarnitsky et al., 2000b, Buratowski, 2009). These proteins have changing affinities for the CTD according to its phosphorylation pattern, thus allowing different proteins to be recruited to the mRNA at different stages of transcription (Komarnitsky et al., 2000b, Darnell, 2013).

1.1.3 pre-mRNA processing

A series of pre-mRNA processing events result in the production of mature translatable mRNA. These events include: the addition of 7-methylguanosine cap at the 5’ end of mRNA, splicing out of introns and the formation of a 3’ poly (A) tail. Historically, it was believed that these pre-mRNA processing events were a linear series of unconnected events; however, emerging evidence indicates that these events are interconnected and can influence the efficiency of one another (Darnell, 2013). It is likely that the dynamic recruitment of the pre-mRNA processing proteins to the RNA pol II CTD coordinates these processing events (Orphanides and Reinberg, 2002, Proudfoot et al., 2002).

The first pre-mRNA processing event to occur is the addition of the 7' methylguanosine cap structure to the 5' end of mRNA. The methyl cap is found on most, if not all, eukaryotic mRNAs. There is considerable evidence that mRNA capping influences the efficiency of other pre-mRNA processing events such as: splicing, polyadenylation and mRNA export (Hocine et al., 2010). The formation of the methyl cap and the role of the methyl cap in gene expression are described in detail later in this introduction.

Splicing is the process by which non-coding sequences (introns) are removed and the coding sequences (exons) are joined together. Splicing is catalysed by the splicesome; which is a large ribonucleoprotein (RNP) complex consisting of U1, U2, U4, U5, U6 smaller nuclear RNPs and an additional 150 proteins (Stark and Luhrmann, 2006). Since several splicing proteins associate with the phosphorylated CTD of RNA pol II, it is likely that splicing occurs predominately co-transcriptionally (Braunschweig et al., 2013, Hsin and Manley, 2012).

The final pre-mRNA processing event is polyadenylation. Polyadenylation is a two-step event which involves the endonucleolytic cleavage of the pre-mRNA at the 3' end, approximately 10-30 nucleotides downstream of a AAUAAA signal sequence, followed by the addition of a poly(A) tail onto the cleaved 3' end (Colgan and Manley, 1997). Polyadenylation is catalysed by a large complex of proteins, of which several are recruited to the pre-mRNA by the CTD of RNA pol II (Shi et al., 2009). Polyadenylation is required for efficient mRNA transcription, mRNA export and mRNA translation (Weill et al., 2012, Hsin and Manley, 2012). Following polyadenylation, the mature mRNA is exported to the cytoplasm for translation.

1.1.4 Translation initiation

The translation of mRNA into protein is the final step in gene expression. mRNA translation consists of three main stages: initiation, elongation and termination. Although each of these stages are regulated to some degree, translation is predominately regulated at the initiation stage in which the 43S pre-initiation complex associates with the methyl cap at the 5' end of mRNA. This association is mediated by the eIF4F complex which consists of: (1) eIF4E, an mRNA 5' cap binding protein, (2) eIF4G, a scaffold protein and (3) eIF4A, an ATP-dependent RNA helicase. The binding of the eIF4F complex to the 5' mRNA methyl cap is essential for the efficient cap-dependent translation of most cellular mRNAs. The 43S pre-initiation complex consists of the 40S ribosomal subunit, the eIF2-GTP-Met-tRNA ternary complex, eIF3, eIF1, eIF1A and most likely eIF5. The recruitment of the 43S pre-initiation complex to mRNA is aided by the association of eIF4G with eIF3 and eIF4E. Once recruited to mRNA, the 43S pre-initiation complex scans the mRNA downstream of the 5' methyl cap until it finds the initiation codon. After the dissociation of several translation factors, the 60S ribosomal subunit joins to form the 80S translation initiation complex and translation elongation subsequently initiates (Jackson et al., 2010, Merrick, 2004, Sonenberg and Hinnebusch, 2009, Gingras et al., 1999).

Although the translation of most mRNAs is dependent on the mRNA methyl structure, a subset of cellular mRNAs can be translated by a distinct mechanism which is independent of the methyl cap. It has been proposed that these mRNAs recruit the 43S pre-initiation complex near, or directly to, the initiation start codon using secondary structures elements within their 5' untranslated region (UTR) called internal ribosomal entry sites (IRES). Several cellular IRES-containing mRNAs have been described and these mRNAs often contain

complex secondary structure in their 5' UTR (Macejak and Sarnow, 1991, Stoneley and Willis, 2004, Johannes et al., 1999). In optimal growth conditions, cap-dependent mRNA translation predominates and IRES-dependent translation is rather inefficient. However, the repression of cap-dependent translation during states of cellular stress results in a large pool of available ribosomes and translation initiation factors to translate IRES-containing mRNAs. Cellular stress conditions which supress mRNA translation include: hypoxia, apoptosis, nutrient limitation and viral infection (Holcik and Sonenberg, 2005, Stoneley and Willis, 2004, Svitkin et al., 2005a, Liwak et al., 2012). Moreover, the availability of eIF4E has been implicated in the switch from cap-dependent to IRES-dependent translation (Braunstein et al., 2007, Miskimins et al., 2001, Dyer et al., 2003, Svitkin et al., 2005b).

Interestingly, many proteins that can be translated via IRES elements are known regulators of cell survival, proliferation or death (Liwak et al., 2012, Komar and Hatzoglou, 2011, Holcik and Sonenberg, 2005, Miskimins et al., 2001). For example the tumour suppressor p53, which regulates cell cycle progression and apoptosis, can be translated in an IRES-dependent manner (Halaby and Yang, 2007, Ray et al., 2006, Yang et al., 2006). However, it is important to note that the full spectrum of endogenous mammalian mRNAs which are translated via an IRES element remains unknown.

The exact mechanisms of cellular IRES-mediated translation remains poorly understood, but it thought that IRES-containing mRNAs can be translated by different mechanisms. That is, IRES-containing mRNAs have different requirements for ITAFs (IRES trans-acting factors), which are RNA-binding proteins that can either enhance or repress IRES activity, and for the canonical translation initiation factors. Potential mechanisms for the translation of cellular

IRES-containing mRNAs include: (1) most, if not all, the canonical translation initiation factors and several ITAF are required; (2) a few canonical factors and ITAFs are required; (3) canonical factors are not necessary, but some ITAFs are required (Komar and Hatzoglou, 2011, Stoneley and Willis, 2004).

1.2 Methyl cap structures

The 7-methylguanosine cap structure (termed Cap0) (Figure 1.1) was first identified on eukaryotic viral mRNA and was subsequently found on eukaryotic mRNA (Furuichi and Miura, 1975, Furuichi et al., 1975). The 7-methylguanosine cap structure consists of an inverted guanosine group that is linked to the first transcribed nucleotide, via a 5'-5' triphosphate linkage, and is methylated at the N-7 position (Figure 1.1). The 7-methylguanosine cap is found at the 5' end of eukaryotic mRNAs and eukaryotic viral RNA, but it is absent from bacterial and archaeal RNA (Furuichi and Shatkin, 2000, Shatkin, 1976, Shuman, 2002).

Additional cap structures have been identified in which the methyl cap structure is further methylated at the 2'-O ribose position of the first and second nucleotide (termed Cap1 and Cap2, respectively) (Figure 1.1). Cap1 and Cap2 containing mRNAs are present in higher eukaryotes (including humans) but are absent in lower eukaryotes (including yeast). Despite the enzymatic activities for Cap1 and Cap2 methylation being identified in human cell extracts over 30 years ago (Langberg and Moss, 1981), the enzymes which catalyse 2'-O ribose methylation in humans were only recently identified (Belanger et al., 2010, Werner et al., 2011). Evidence indicates that 2'-O-ribose methylation of the first transcribed nucleotide occurs in the nucleus, whereas 2'-O-ribose methylation of the second nucleotide occurs in the cytoplasm (Langberg and Moss, 1981,

Furuichi and Shatkin, 2000). The exact mechanisms by which Cap1 and Cap2 are formed and the biological significance of these cap structures remain to be elucidated.

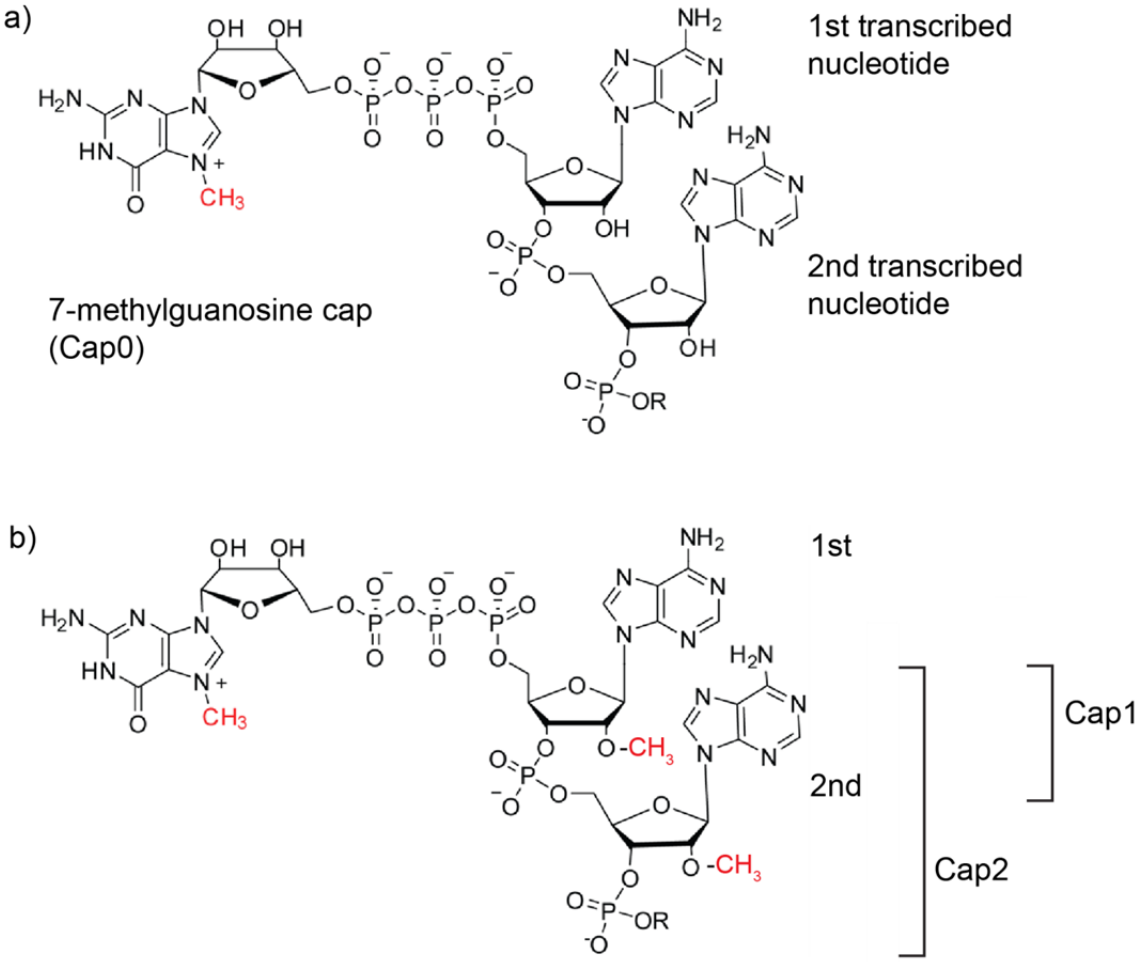


Figure 1.1 The methyl cap structures (Cap0, Cap1, Cap2) of eukaryotic mRNA

a) The 7-methylguanosine cap (Cap0) consists of an inverted guanosine group linked to the first transcribed nucleotide via a 5'-5' triphosphate linkage and is methylated at the N-7 position (shown in red).

b) Cap1 and Cap2 are methylated at the N-7 position and are further methylated at the 2-0' ribose of the first and second nucleotide, respectively.

1.3 Enzymology of 7-methylguanosine cap formation

The 7-methylguanosine cap structure at the 5' end of mRNA is formed by three sequential enzymatic activities. Firstly, the 5' RNA triphosphatase hydrolyses the terminal phosphate from the 5' nascent RNA to produce a diphosphate end. Next, the RNA guanylyltransferase catalyses the addition of a guanosine monophosphate (GMP) to the diphosphate RNA end via a two-step reaction. In the first step, the enzyme reacts with the α phosphate of guanosine triphosphate (GTP) to form a covalent GMP-enzyme complex. In the second step, the enzyme adds the GMP to the diphosphate RNA end to form the guanosine cap. To finalise the 7-methylguanosine structure, the RNA methyltransferase transfers a methyl group from S-adenosylmethionine (SAM) to the N-7 position of the guanosine cap and releases S-adenosylhomocysteine (SAH) (Shuman, 2002, Furuichi and Shatkin, 2000). The enzymology of 7-methylguanosine cap formation is shown in Figure 1.2.

The biochemical reactions involved in 7-methylguanosine cap (methyl cap) formation were initially elucidated using viral capping systems (Furuichi and Shatkin, 2000, Shuman, 2002, Shatkin, 1976), but for the purpose of this thesis, only eukaryotic capping systems will be described further in this introduction. The three enzymatic activities (triphosphatase, guanylyltransferase, methyltransferase) required for the formation of the methyl cap are conserved across all eukaryotes, but there are differences in the way the enzymes are configured. The capping enzymes found in *Saccharomyces cerevisiae, Schizosaccharomyces pombe* and *Homo sapiens* are summarised in Table 1.1. In lower eukaryotes, such as yeast, the three activities are found in three distinct proteins (Table 1.1) (Shibagaki et al., 1992, Tsukamoto et al., 1997, Mao et al., 1995, Shuman et al., 1994, Pei et al., 2001). Whereas, in higher

eukaryotes, such as mammals and other metazoa, the RNA triphosphatase and guanylyltransferase activities reside in a bi-functional protein and the RNA methyltransferase activity reside in a separate protein. In humans, the capping enzyme (RNGTT) contains both RNA triphosphatase and guanylyltransferase activities and RNA guanine 7-methyltransferase (RNMT) contains methyltransferase activity (Yokoska et al., 2000, Tsukamoto et al., 1998a, Tsukamoto et al., 1998b, Yue et al., 1997, Pillutla et al., 1998a, Shuman, 2002, Saha et al., 1999).

The capping enzymes in yeast and humans are essential for methyl cap formation and cell viability (Wang and Shuman, 1997, Mao et al., 1995, Tsukamoto et al., 1997, Chu and Shatkin, 2008, Shafer et al., 2005b). The fact that the yeast capping enzymes can be functionally replaced by the mammalian capping enzymes suggests that the capping enzymes are functionally conserved from yeast to humans (Saha et al., 1999, Yue et al., 1997).

The entire 7-methylguanosine cap structure can be removed by several well-characterised decapping enzymes (Chang et al., 2012, Wang et al., 2002, Song et al., 2010, Arribas-Layton et al., 2013). To date, there is no experimental evidence to suggest that the methyl cap can be demethylated and that a cap demethylase exists.

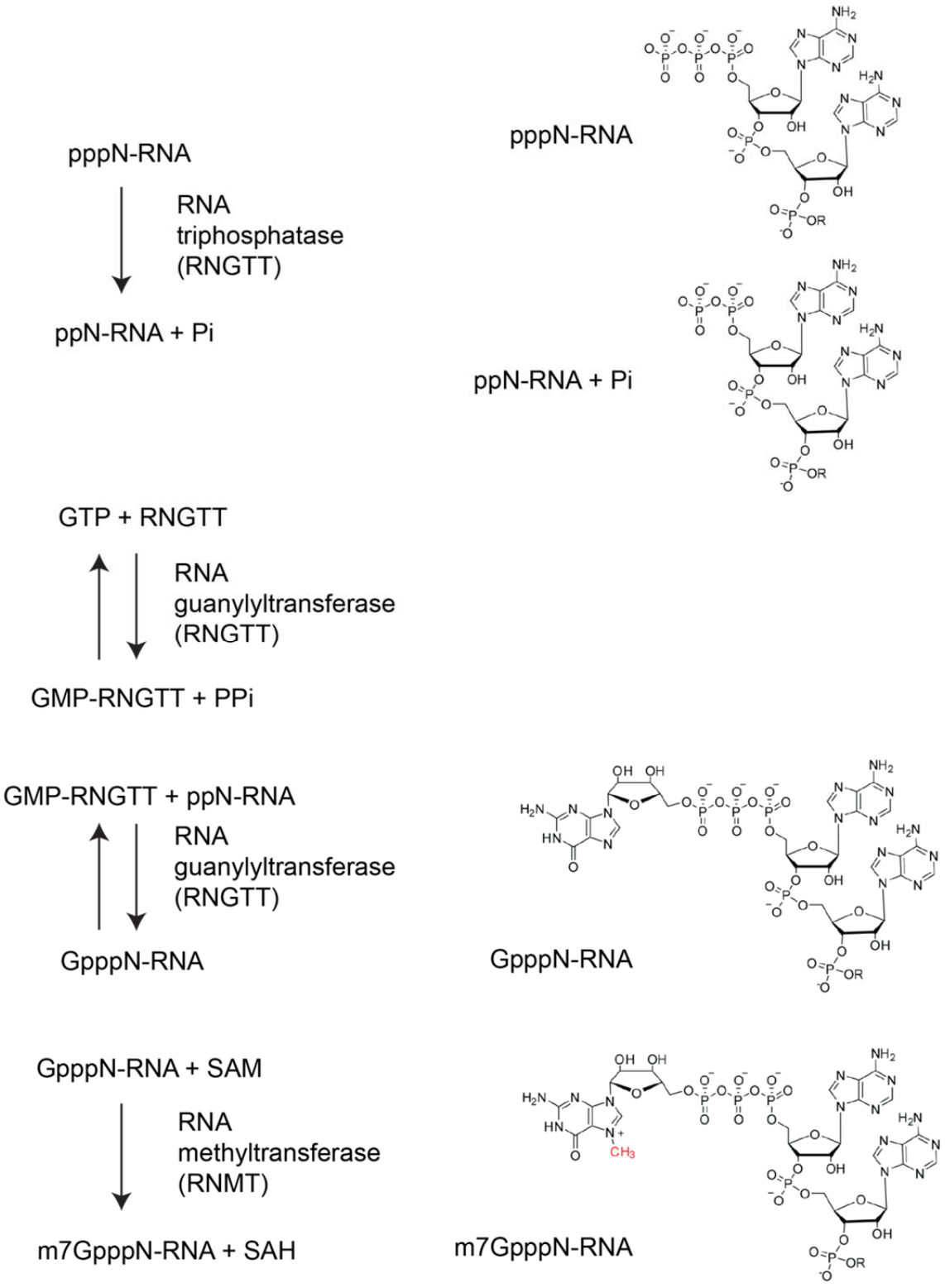


Figure 1.2 Enzymatic synthesis of the mRNA 7-methylguanosine cap

The synthesis of the 7-methylguanosine cap is described in the text. The enzymatic reactions are shown on the left and the reactions are graphically illustrated on the right. RNGTT and RNMT are the human capping enzymes. RNGTT contains both triphosphatase and guanylyltransferase activity. RNMT contains methyltransferase activity. The guanylyltransferase reaction is reversible, but the methyltransferase and triphosphatase reactions are not.

Enzymatic activities	*S. pombe*	*S. cerevisiae*	*H. sapiens*
RNA triphosphatase	Pct1p	Cet1p	RNGTT (bifunctional enzyme)
RNA guanylyltransferase	Pce1p	Ceg1p	RNGTT (bifunctional enzyme)
RNA methyltransferase	Pcm1p	Abd1	RNMT

Table 1.1 Enzymes which synthesise the 7-methylguanosine cap in yeast and humans

The 7-methylguanosine cap in eukaryotes is formed by three sequential enzymatic activities (RNA triphosphatase, RNA guanylyltransferase and RNA methyltransferase). The proteins which contain these activities in *Homo sapiens* and the yeast species *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* are indicated.

1.4 The human mRNA cap methyltransferase complex

As mentioned above, the final step in methyl cap formation is the transfer of a methyl group from SAM to the guanosine cap at the N-7 position (Figure 1.2). Human RNA cap methyltransferase activities were first isolated from HeLa cells extracts (Ensinger and Moss, 1976) and RNMT, the human RNA cap methyltransferase, was subsequently identified based on sequence homology to Abd1, the *S. cerevisiae* cap methyltransferase (Pillutla et al., 1998b, Tsukamoto et al., 1998b). RNMT is only able to methylate a GTP group attached to RNA (Cowling lab, unpublished) and RNMT expression is essential for cellular cap methylation. Since RNMT expression is required for efficient gene expression and cell viability, it is likely that there are no other cellular RNA

methyltransferases that can compensate for the loss of cellular RNMT (Chu and Shatkin, 2008, Shafer et al., 2005a, Aregger and Cowling, 2013, Cowling, 2009a, Gonatopoulos-Pournatzis et al., 2011).

Our laboratory recently discovered that RNMT exists in a complex with a novel protein RAM (RNMT activating-mini protein). This RNMT-RAM hetero-dimer complex is termed the human mRNA cap methyltransferase complex (Figure 3). It has been observed that RAM enhances RNMT activity *in vitro* up to 5-fold and RAM expression is essential for cellular cap methylation, efficient gene expression and cell viability. Moreover, it has been shown that RAM is an RNA-binding protein and promotes the recruitment of RNA to RNMT(Gonatopoulos-Pournatzis et al., 2011). RNMT and RAM are predominately localised to the nucleus (Gonatopoulos-Pournatzis and Cowling, 2014, Gonatopoulos-Pournatzis et al., 2011). RNMT contains three nuclear localisation signals and RAM contains two PY nuclear localisation signals, which ensure their correct cellular localisation (Gonatopoulos-Pournatzis et al., 2011, Shafer et al., 2005b, Wen and Shatkin, 2000, Gonatopoulos-Pournatzis and Cowling, 2014). It has been shown that a cytosolic enzymatically active mutant of RNMT is unable to rescue loss of cell viability caused by depletion of endogenous RNMT. Hence, the nuclear localisation of RNMT is necessary for its cellular functions (Shafer et al., 2005a).

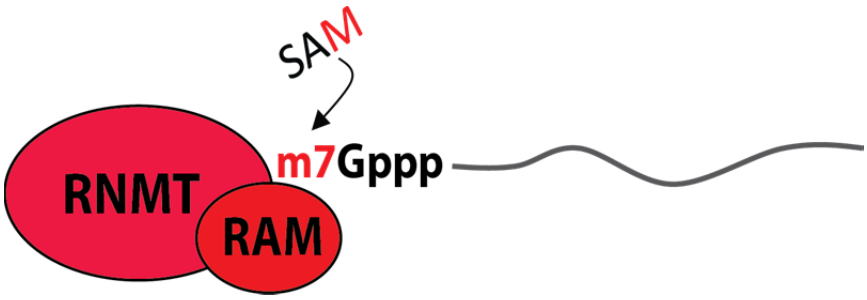


Figure 1.3 The human mRNA cap methyltransferase complex

The human mRNA cap methyltransferase complex consists of RNMT (which contains RNA cap methyltransferase activity) and its activating subunit RAM (RNMT-activating miniprotein). RNMT catalyses the transfer of a methyl group from SAM to the N-7 position of the guanosine cap.

1.5 7-methylguanosine cap formation occurs co-transcriptionally

There is increasing evidence that formation of the cap occurs predominately, if not entirely, co-transcriptionally (Figure 1.4). Data suggest that nascent RNA is capped and subsequently methylated as soon as it emerges from RNA pol II (Furuichi and Shatkin, 2000). As described above, RNA pol II is recruited to genes with the CTD hypophosphorylated and it is phosphorylated during, or soon after, transcription initiation (Cadena and Dahmus, 1987, Laybourn and Dahmus, 1990, Lu et al., 1991). CTD serine 5 phosphorylation is associated with the initiation of transcription, whereas CTD serine 2 phosphorylation is primarily associated with transcription elongation (Marshall et al., 1996, Komarnitsky et al., 2000b, Trigon et al., 1998). Evidence suggest that the phosphorylated CTD acts as a binding platform for the mRNA capping enzymes, thereby recruiting these proteins to the nascent transcript (Komarnitsky et al., 2000b, Buratowski, 2009). Several studies have shown that the methyl cap is formed very early in transcription and it is thought that the

capping enzymes are recruited during, or soon after, transcription initiation (Rasmussen and Lis, 1993, Mandal et al., 2004b, Chiu et al., 2002, Glover-Cutter et al., 2008a, Schroeder et al., 2000, Komarnitsky et al., 2000b). The fact that methyl cap formation is faster on RNA that is being actively transcribed, compared to RNA alone, suggests that mRNA capping and transcription initiation are functionally coupled (Moteki and Price, 2002). The CTD of RNA II plays an important role in the formation of the methyl cap. For example, it has been shown that RNA Pol II is essential for efficient mRNA capping and the phosphorylated CTD enhances the recruitment of the capping enzymes to nascent RNA. (McCracken et al., 1997). Moreover, the phosphorylated CTD of RNA Pol II has been demonstrated to interact with the triphosphatase and methyltransferase in yeast (McCracken et al., 1997, Cho et al., 1997) and the mammalian capping enzyme (Yue et al., 1997, Ho et al., 1998, Ghosh et al., 2011, McCracken et al., 1997). A direct interaction between the mammalian cap methyltransferase and RNA Pol II remains controversial. Although it has been reported that RNMT does not interact directly with RNA Pol II (Shatkin and Manley, 2000, Pillutla et al., 1998b), recent experiments in our laboratory have demonstrated an interaction (Cowling, unpublished). Moreover, one study reported that RNMT, the human cap methyltransferase, associates with RNA Pol II via an interaction with the capping enzyme (Pillutla et al., 1998b). A recent study from our lab has demonstrated the N-terminus non-catalytic domain of RNMT mediates its recruitment to transcription initiation sites. It was observed that the recruitment of RNMT to chromatin is inhibited by 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB), a RNA Pol II phosphorylation inhibitor, which suggests that RNMT interacts with RNA pol II at least indirectly (Aregger

and Cowling, 2013). The precise mechanisms by which RNMT is recruited to either transcription initiation sites, or to the transcripts, remains to be elucidated.

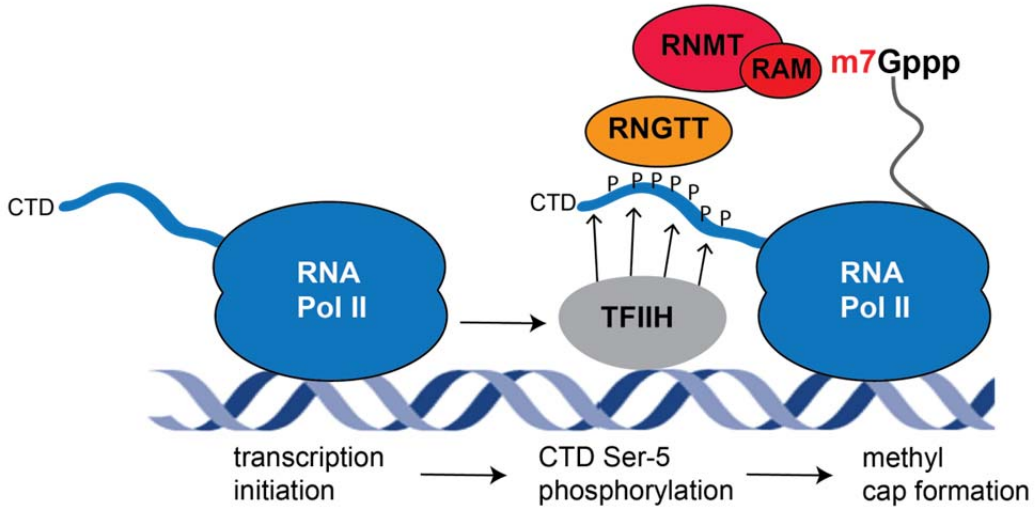


Figure 1.4 7-methylguanosine cap formation occurs co-transcriptionally

RNA pol II is recruited to genes with its CTD hypophosphorylated. Shortly after the initiation of transcription, TFIIH phosphorylates the CTD on Ser-5. It is thought that the capping enzymes (RNGTT and RNMT) associate with the phosphorylated CTD, thereby recruiting these enzymes to the 5' end of nascent RNA. RNGTT (human capping enzyme) and RNMT-RAM (human mRNA cap methyltransferase complex).

1.6 The regulation of mRNA cap methylation

The formation of the methyl cap was long thought to be a constitutive process, however, emerging evidence indicates that it is regulated (Ho and Shuman, 1999, Wen and Shatkin, 2000, Wen and Shatkin, 1999b, Schwer et al., 2000, Cowling and Cole, 2007, Fernandez-Sanchez et al., 2009a, Cole and Cowling, 2009). For the purpose of this introduction only the regulation of mRNA cap methylation will be described further. During mRNA cap methylation, the RNA methyltransferase catalyses the transfer of a methyl group from the methyl

donor SAM to the N-7 position of the guanosine cap. Known regulators of mRNA cap methylation either increase the recruitment of the methyltransferase to the transcript, or increase the efficiency of the methyltransferase capping reaction (Cowling, 2009b).

Since transcripts lacking the methylated cap accumulate under amino acid and glucose starvation in *S. cerevisiae*, it is possible that nutrient availability regulates mRNA cap methylation in yeast (Jiao et al., 2010). The human transcription factors E2F1 and c-Myc, which stimulate cell growth in response to growth factors, have been observed to increase cap methylation on specific human transcripts (Figure 1.5) (Fernandez-Sanchez et al., 2009b, Cole and Cowling, 2009, Cowling and Cole, 2007, Aregger and Cowling, 2012). The Myc proteins (c-Myc and N-Myc) are known to be essential for cell growth and proliferation. c-Myc is a well-established oncoprotein and its expression is deregulated in an estimated 70% of human cancers. The c-Myc proteins are transcription factors which regulate transcription in a gene-specific manner; typically they repress, or activate, genes by 1.5-2-fold (Dang, 2012, Felsher and Bishop, 1999, Cowling and Cole, 2006). Myc promotes the cap methylation of several transcripts; including most Myc transcriptionally regulated genes, as well as some non-transcriptionally regulated genes. Interestingly, the upregulation of mRNA cap methylation by c-Myc is independent of its ability to transcriptionally regulate genes (Cowling and Cole, 2007). As shown in Figure 1.5, Myc upregulates cap methylation by two distinct mechanisms. The first mechanism involves an upregulation of RNA Pol II CTD phosphorylation (serine 5). As described earlier, the kinase subunit of TFIIH phosphorylates the CTD of RNA Pol II at serine 5. It has been observed that Myc binds directly to TFIIH and enhances its recruitment to transcription initiation sites, thus increasing

serine 5 CTD phosphorylation and enhancing the recruitment of the capping enzymes. For the transcripts tested, this correlates with enhanced 7-methylguanosine cap formation and protein translation (Cowling and Cole, 2007). It is likely that any protein which upregulates serine 5 CTD phosphorylation will enhance the formation of the mRNA methyl cap.

The second mechanism by which c-Myc upregulates cap methylation involves the increased expression of the enzyme S-adenosyl homocysteine hydrolase (SAHH). All methylation reactions convert SAM into S-adenosyl homocysteine (SAH), which inhibits methylation reactions (Chiang et al., 1996). Our laboratory discovered that Myc upregulates the expression of SAHH, an enzyme which catalyses the hydrolysis of SAH into adenosine and homocysteine, thus neutralising the inhibitory effect of SAH. The upregulation of SAHH by c-Myc is essential for Myc-induced mRNA cap methylation, protein synthesis, cell proliferation and cell transformation. These observations demonstrate that Myc-induced methyl cap formation is a crucial function of Myc. Strikingly, it was observed that inhibition of cap methylation selectively inhibits the proliferation of cells with elevated c-Myc expression, suggesting that targeting mRNA cap methylation could be an effective strategy to inhibit upregulated c-Myc function in cancer cells (Fernandez-Sanchez et al., 2009a).

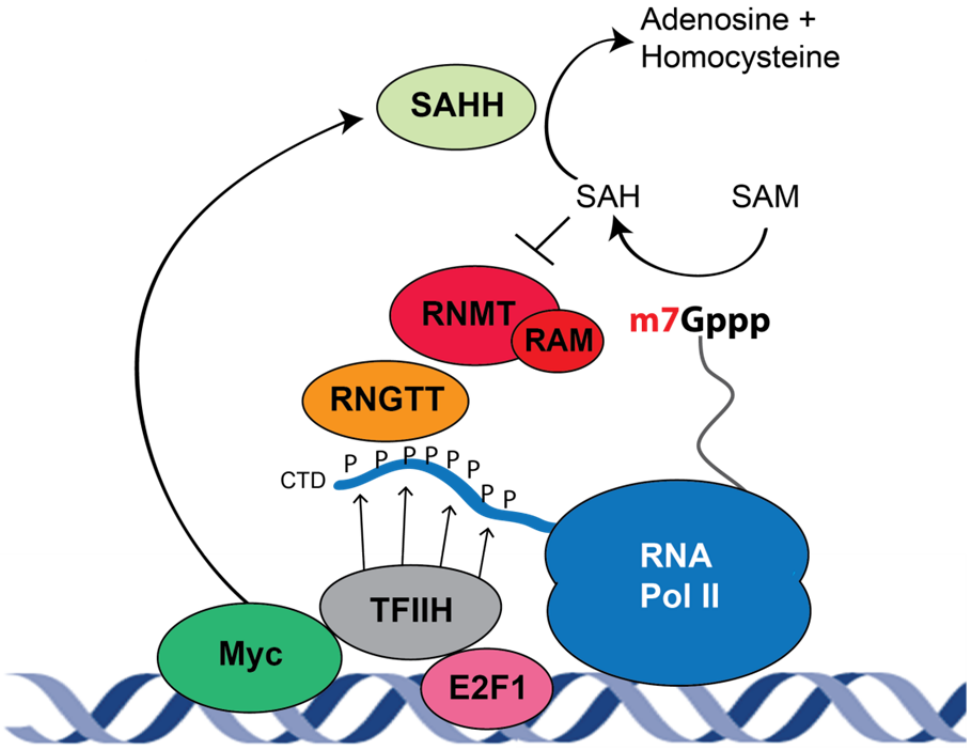


Figure 1.5 Myc and E2F1 upregulate 7-methylguanosine cap formation

The 7-methylguanosine cap is formed co-transcriptionally. Shortly after the initiation of transcription, TFIIH phosphorylates the CTD on Ser 5. It is thought the capping enzymes interact with the phosphorylated CTD, thereby recruiting these enzymes to the 5' end of nascent RNA. Myc and E2F1 upregulate methyl cap formation by enhancing the recruitment of TFIIH. Myc can also promote methyl cap formation via a second mechanism, whereby Myc enhances the expression of SAHH, which removes the inhibitory by-product of cap methylation, SAH. SAH is hydrolysed into adenosine and homocysteine.

1.7 Functions of the 7-methylguanosine cap in gene expression

The 7-methylguanosine cap structure at the 5’ end of mRNA is essential for cell viability and efficient gene expression. The methyl cap is known to promote several steps in the gene expression pathway. For example, the methyl cap is essential for the translation of most mRNAs and it is reported to protect mRNA from degradation and to enhance transcription, splicing, polyadenylation and mRNA nuclear export (Figure 1.6) (Furuichi and Shatkin, 2000). The functions of the methyl cap are mostly mediated by proteins which bind the mRNA methyl cap. The cap binding complex (CBC) and eukaryotic translation initiation factors 4E (eIF4E) are the most well-characterised cap binding proteins (Topisirovic et al., 2011). Although, it is important to note that other cap binding proteins have been reported, such as poly(A)-specific ribonuclease and pumilio2 (Dehlin et al., 2000, Gao et al., 2000, Martínez et al., 2000, Cao et al., 2010).

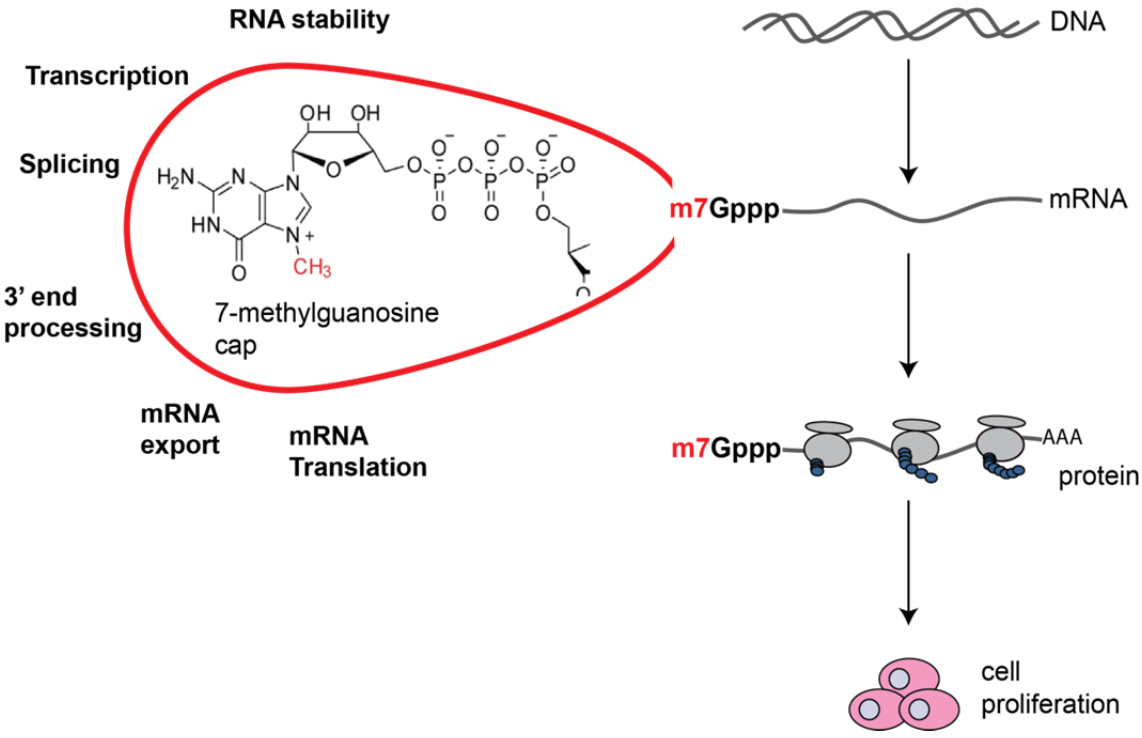


Figure 1.6 Functions of the 7-methylguanosine cap in gene expression

The 7-methylguanosine cap is essential for the translation of most mRNAs and for efficient gene expression and cell proliferation. This schematic illustrates the main functions of the 7-methylguanosine cap in gene expression. These functions include promoting RNA stability, mRNA transcription, pre-mRNA splicing, pre-mRNA 3' end processing, mRNA export and mRNA translation. These functions of the cap are predominately mediated by two cap-binding proteins: eIF4E and CBC (described in detail in the text).

1.7.1 The 7-methylguanosine cap stabilises RNA

Soon after the discovery of the 7-methylguanosine cap it was shown that the methyl cap functions to stabilise RNA. Methyl capped RNA is more stable, compared to uncapped RNA, when microinjected into *X. laevis,* or incubated with wheat germ extracts (Furuichi et al., 1977, Green et al., 1983, Shimotohno et al., 1977). The mRNA methyl cap structure promotes RNA stability by acting as a blocking structure, thereby protecting the transcript from exoribonucleolytic degradation (Shimotohno et al., 1977, Inoue et al., 1989, Murthy et al., 1991).

Although the methyl cap has been shown to stabilise transcripts, this function is not observed in all cellular systems. It is likely that the role of the methyl cap in mRNA stability is species specific, or gene specific (Grudzien et al., 2006, Schwer et al., 2000, Furuichi et al., 1977, Fresco and Buratowski, 1996, Schwer and Shuman, 1996, Schwer et al., 1998). Moreover, the cap-binding proteins CBC and eIF4E function to further enhance the stability of RNA by competing with the decapping enzymes for binding to the methyl cap (Jiao et al., 2013, Grudzien et al., 2006, Gonatopoulos‐Pournatzis and Cowling, 2014, Schwartz and Parker, 2000).

1.7.2 The cap-binding proteins

1.7.2.1 The cap-binding complex

The cap-binding complex (CBC) is a hetero-dimer composed of the subunits CBP20 and CBP80. The CBC was first purified from cell extracts on the basis of its ability to specifically bind the 7-methylguanosine cap at the 5’ end of mRNA (Izaurralde et al., 1994, Izaurralde et al., 1995). Although structural and mutational studies have demonstrated that the CBP20 subunit contains the methyl cap binding pocket, both subunits of the CBC are required for binding to the methyl cap at the 5’end of mRNA (Izaurralde et al., 1994, Izaurralde et al., 1995, Kataoka et al., 1995, Calero et al., 2002, Mazza et al., 2001). The CBC binds to the methylated cap with greater affinity compared to the non-methylated cap, which suggests that cap methylation is important for efficient binding of the methyl cap to CBC. It is known that the CBC is required for cell growth and promotes several steps in gene expression (Fortes et al., 1999, Das et al., 2000, Narita et al., 2007). Since several studies have observed that the

CBC recruits several pre-mRNA processing proteins to the transcript (Pabis et al., 2013, Narita et al., 2007, Merz et al., 2007, Flaherty et al., 1997), it is likely that the CBC complex predominately mediates its effect on gene expression through the recruitment of other proteins. As shown in Figure 1.7, several functions of the 7-methylguanosine cap are mediated by the CBC.

1.7.2.2 The eukaryotic translation initiation factor 4E (eIF4E)

The translation of most mRNAs is dependent on the 7-methylguanosine cap structure at the 5' end of mRNA. As described above, the main regulatory step in cap-dependent translation initiation is the association of the 43S pre-initiation complex with the 7-methylguanosine cap. This association is mediated by the eIF4F complex which consists of (1) eIF4E, the cap-binding protein, (2) eIF4G, the scaffold protein and (3) eIF4A, the ATP-dependent RNA helicase (Sonenberg and Hinnebusch, 2009, Gingras et al., 1999). eIF4E is a 24kDa protein that specifically binds the methyl cap, thereby recruiting the eIF4F complex to the 5' end of the mRNA. The structure of the mammalian eIF4E bound to the 7-GpppG cap analogue has been determined by X-ray crystallography and biophysical methods (Niedzwiecka et al., 2002, Marcotrigiano et al., 1997). The methyl cap binds to eIF4E with much higher affinity compared to the non-methylated cap, which indicates that cap methylation is important for the eIF4E-methyl cap interaction (Niedzwiecka et al., 2002, von der Haar et al., 2004).

There is considerable evidence that expression of eIF4E is rate-limiting for cap-dependent translation (De Benedetti et al., 1991, Duncan et al., 1987, Hiremath et al., 1985). The interaction between eIF4E and the methyl cap enhances

mRNA translation by promoting the recruitment of translation machinery to the 5' end of mRNA (Sonenberg et al., 1979, Sonenberg et al., 1980). eIF4E is an essential component of the translation machinery, but its overexpression only modestly increases global protein synthesis, while strongly enhancing the translation of a subset of eIF4E-dependent mRNAs (Koromilas et al., 1992, De Benedetti and Harris, 1999, Rosenwald et al., 1993, Shantz and Pegg, 1994, Kevil et al., 1995). Several studies have shown eIF4E overexpression to be oncogenic both *in vitro* and *in vivo* (Lazaris-Karatzas et al., 1990, De Benedetti and Rhoads, 1990, De Benedetti and Graff, 2004, Ruggero et al., 2004, Wendel et al., 2004b). As shown in Figure 1.7, eIF4E mediates several functions of the methyl cap. The regulation of eIF4E activity and the role of eIF4E in cancer will be described in section 1.9.1.

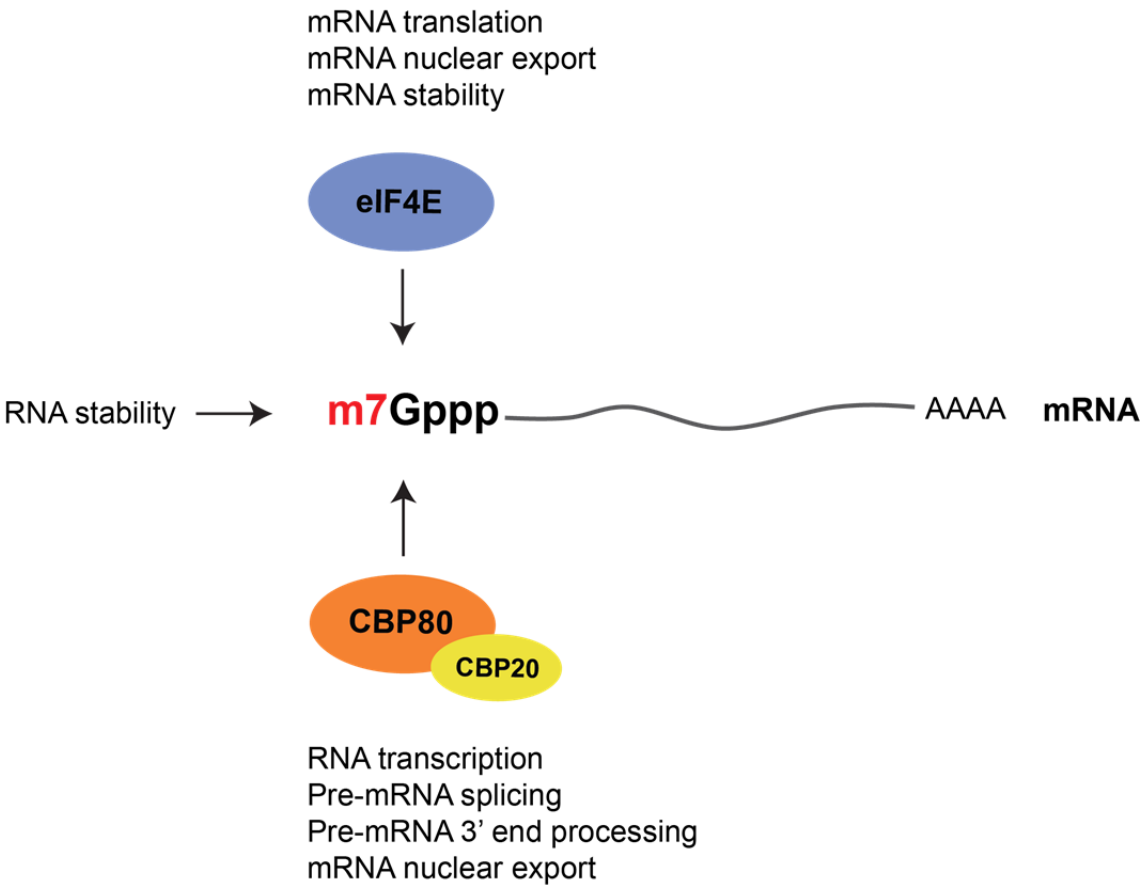


Figure 1.7 Cap-binding proteins mediate most of the functions of the 7-methylguanosine cap

Most of the functions of the 7-methylguanosine cap are mediated by two cap-binding proteins: CBC and eIF4E. CBC (CBP20 and CBP80) promotes transcription, pre-mRNA splicing, pre-mRNA 3' end processing and mRNA nuclear export. eIF4E binding to the 7-methylguanosine cap is essential for the translation of most mRNAs. eIF4E also promotes mRNA stability and nuclear export. The 7-methylguanosine cap structure itself stabilises RNA.

1.7.3 The 7-methylguanosine cap is required for cap-dependent translation

From yeast to humans, the methyl cap structure is essential for the translation of most mRNAs. Soon after the discovery of the methyl cap, *in vitro* experiments demonstrated that mRNA translation is dependent on the methyl cap structure (Muthukrishnan et al., 1975, Zan-Kowalczewska et al., 1977, Shimotohno et al., 1977). The methyl cap has also been shown to be essential

for mRNA translation *in vivo*. Methyl capped RNA injected into *X. laevis* oocytes are translated more efficiently compared to guanosine capped RNA and this effect was not due to increased transcript stability (Gillian-Daniel et al., 1998, Drummond et al., 1985).The binding of eIF4E to the methyl cap is essential for efficient cap-dependent translation (Sonenberg et al., 1978, Sonenberg et al., 1979, Altmann et al., 1985, Altmann et al., 1987, Sonenberg et al., 1980). Since the methylation of the guanosine cap enhances eIF4E binding to the methyl cap, it is likely that cap methylation promotes mRNA translation. Indeed, the methylation of the guanosine cap is essential for the translation of mRNA in cell-free systems (Both et al., 1975, Weber et al., 1977, Filipowicz, 1978). Moreover, inactivation of the RNA methyltransferase in yeast leads to a robust decrease in protein synthesis without a corresponding decrease in transcript stability (Schwer et al., 2000*).* Although it has been reported that depletion of cellular RNMT does not significantly impact global protein synthesis, it was found that a subset of mammalian transcripts are highly dependent on mRNA cap methylation for their translation (Cowling, 2009a). The full spectrum of mRNAs which are highly dependent on cap methylation for their translation remains to be elucidated.

1.7.5 The 7-methylguanosine cap promotes transcription

The transcription of eukaryotic genes is a multistep process in which there are multiple opportunities for regulation. RNA pol II pauses at promoter-proximal sites shortly after the initiation of transcription, and this pausing event is important for the control of transcription (Guenther et al., 2007, Rahl et al., 2010). Since the recruitment of the capping enzymes coincide with this pausing,

transcription and the formation of the methyl cap are functionally linked. Experiments carried out in the yeast, or *in vitro* using human proteins, have observed that the capping enzyme promotes transcription and can interact with proteins that function in transcription (Pei and Shuman, 2002, Lindstrom et al., 2003, Wen and Shatkin, 1999a, Mandal et al., 2004a). Several lines of evidence suggest that the RNA methyltransferase can influence transcription. For example, the cap methyltransferase in yeast has been shown to be required for the transcription of a subset of genes (Schroeder et al., 2004) and to recruit P-TEFb to chromatin, which alleviates RNA pol II pausing on chromatin (Guiguen et al., 2007). In both yeast, and humans, the RNA methyltransferase co-localises with paused RNA pol II, and is present both at gene promoters and throughout the gene body (Glover-Cutter et al., 2008b, Komarnitsky et al., 2000a, Schroeder, 2000). Since cap methylation is not required for the transcription of a subset of genes in yeast, it is likely that cap methylation enhances transcription in a gene-specific manner (Schroeder et al., 2004). However, no studies to date, have established how RNMT, the human cap methyltransferase, affects the human transcriptome.

In addition, the methyl cap has the potential to influence transcription via the CBC. The CBC, which is located at the 5' ends of genes and throughout gene bodies, promotes transcription in both yeast and mammalian cells (Hossain et al., 2013, Listerman et al., 2006, Lahudkar et al., 2011, Lenasi et al., 2011).

1.7.6 The 7-methylguanosine cap is required for efficient pre-mRNA splicing

The incubation of *in vitro* transcribed methyl-capped or uncapped RNA with cell extracts demonstrated that the methyl cap is required for efficient splicing (Konarska et al., 1984, Edery and Sonenberg, 1985, Ohno et al., 1987, Patzelt et al., 1987, Izaurralde et al., 1994). *In vivo* experiments carried out in *X. laevis* have observed that microinjected methyl-capped RNA is spliced more efficiently than uncapped RNA. The splicing of the 5' proximal intron is reported to be highly dependent on the methyl cap structure (Inoue et al., 1989, Ohno et al., 1987). Extracts in yeast have shown that the methyl cap structure promotes splicing in a gene-specific manner (Fresco and Buratowski, 1996, Schwer and Shuman, 1996). Moreover, a recent study revealed that for human endogenous transcripts the methyl cap not only enhances the splicing of the 5' proximal intron, but also promotes the splicing of subsequent downstream introns (Jiao et al., 2013). The full spectrum of mammalian endogenous genes that are dependent on the methyl cap for splicing remains to be determined.

Incubation of *in vitro* transcribed RNA with the methyl-cap analogues m7Gppp and m7GpppG inhibited splicing more efficiently than the cap analogue GpppG, which indicates that the effect of the methyl cap on splicing is mediated by a methyl cap binding protein (Patzelt et al., 1987, Izaurralde et al., 1994). The CBC has been shown to mediate the effects of the methyl cap on splicing. Cell extracts depleted of CBC have reduced splicing activity (Izaurralde et al., 1994, Lewis et al., 1996, Izaurralde et al., 1995). Furthermore, inhibition of the interaction between the CBC and methyl capped RNA in *X. laevis* reduced splicing (Izaurralde et al., 1995).

1.7.7 The methylguanosine cap promotes pre-mRNA 3' end processing

The methyl cap influences 3' end processing (which involves endonucleolytic cleavage at the poly(A) site followed by the addition of the poly(A) tail). Experiments *in vitro* and in *X. laevis* have shown that the methyl cap promotes endonucleolytic cleavage (Georgiev et al., 1984, Cooke and Alwine, 1996, Gilmartin et al., 1988). Furthermore, in mammalian cells, methyl capped RNA is cleaved at the poly (A) site much more efficiently than uncapped RNA (Jiao et al., 2010). Since the mammalian capping enzymes (RNGTT and RNMT) are found to be located at both the 5' and the 3' ends of genes, it is possible that the capping enzymes influence pre-mRNA 3' end processing. The fact that a m7G cap analogue inhibits 3' end processing, suggests that a cap binding protein is required for 3' end processing (Glover-Cutter et al., 2008a). Indeed, the CBC is required for efficient 3' pre-mRNA processing in mammalian cells (Flaherty et al., 1997), but the precise mechanism of this remains to be elucidated.

1.7.8 The 7-methylguanosine cap promotes nuclear export of mRNA

The effective nuclear export of microinjected mRNA and snRNA is dependent of the methyl cap structure and this effect is mostly mediated by the CBC (Izaurralde et al., 1992, Izaurralde et al., 1995, Cheng et al., 2006, Dargemont and Kuhn, 1992, Jarmolowski et al., 1994). eIF4E has been observed to promote mRNA export in a gene-specific manner and eIF4E-mediated nuclear export of mRNA is dependent of the ability of eIF4E to bind the methyl cap (Culjkovic et al., 2005, Rousseau et al., 1996b, Cohen et al., 2001, Strudwick and Borden, 2002). The full spectrum of mammalian transcripts that are dependent on the methyl cap for nuclear export remains to be elucidated.

1.8 Breast cancer

1.8.1 Introduction to cancer

Cancers are diseases characterised by the uncontrolled expansion of clonal cells. In a landmark paper in 2000, Hanahan and Weinberg proposed that all human cancers acquire six hallmark capabilities during cancer development. These hallmarks include: sustaining proliferative signalling, evading growth suppressors, resisting cell death, activating invasion and metastasis, enabling replicative immortality and inducing angiogenesis (Hanahan and Weinberg, 2000). Recently they proposed an additional two hallmark capabilities: reprogramming of energy metabolism and evading immune destruction (Hanahan and Weinberg, 2011). The eight hallmarks of cancer are shown in Figure 1.8. Although it is believed that cells must acquire these hallmarks to achieve a neoplastic state, cells will vary in the mechanism and ordering by which they acquire the hallmarks. For example, in some human cancers, the acquisition of one particular oncogenic genetic mutation may confer numerous capabilities simultaneously (Hanahan and Weinberg, 2011, Hanahan and Weinberg, 2000).

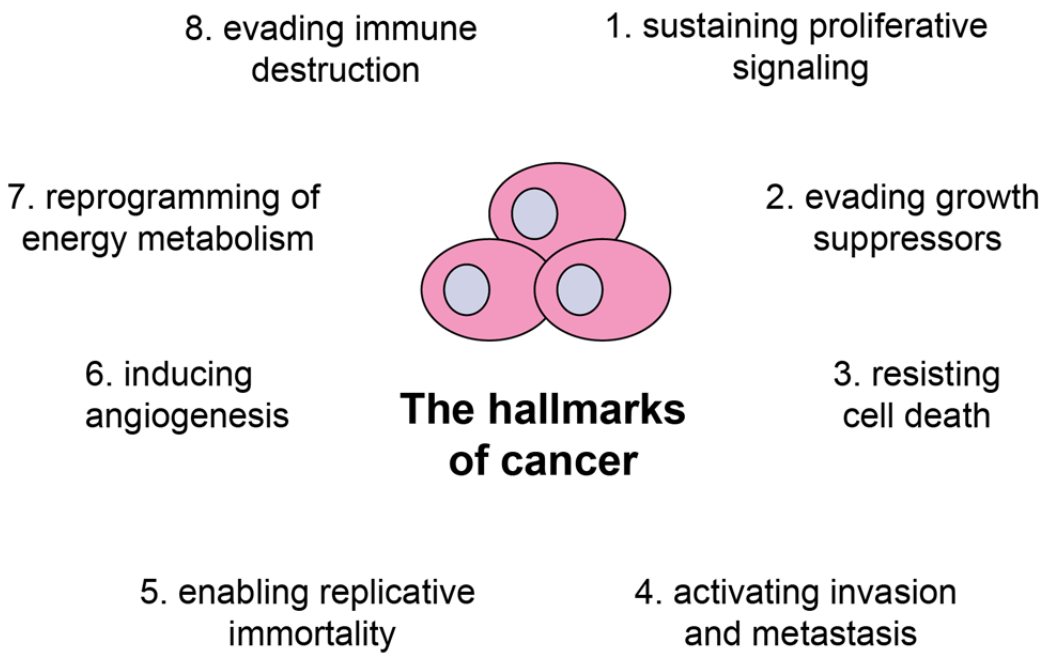


Figure 1.8 The hallmarks of cancer

The eight hallmarks of human cancers. Adapted from (Hanahan and Weinberg, 2011, Hanahan and Weinberg, 2000).

One of the fundamental properties of cancer cells is their ability to proliferate uncontrollably. The growth of normal cells is carefully controlled by the production and release of growth signals, which regulate the cell growth and division cycle. These growth signals bind to and activate cell-surface receptors such as receptor tyrosine kinases (RTKs). These activated receptors are then able to release signals via cellular signalling pathways, which regulate key cellular processes such as cell growth and cell division. Cancer cells are able to induce and sustain hyper-activated proliferative signalling via a number of different mechanisms. For example, they can evade growth factor control via autocrine signalling whereby they can produce growth factors themselves, thus self-activating their own receptors. Alternatively, mutations in components of the signalling pathway that are downstream of the receptor can lead to constitutive

pathway activation; thereby relieving the need to activate these growth pathways via receptor activation. Cancer cells also have mechanisms to evade tumour suppressor proteins, which negatively regulate cell proliferation (Hanahan and Weinberg, 2000, Hanahan and Weinberg, 2011). For example, several cancer genetic studies have shown that tumour suppressors proteins, such as TP53 and PTEN, are frequently mutationally inactivated in several types of human cancers, such as breast cancer (Sorlie et al., 2001, Carey et al., 2006, Kan et al., 2010).

The acquisition of the “hallmarks of cancer” is largely dependent on a succession of mutations in the cancer genome. The multistep development of cancer involves successive rounds of clonal expansion; with each round being triggered by the acquisition of a genetic mutation which confers a selective advantage (Lengauer et al., 1998, Hanahan and Weinberg, 2011). It is known that not all genetic mutations contribute to cancer development. In fact the progression of cancer is a complex interplay of advantageous “driver” mutations, neutral “passenger” mutations and deleterious mutations, as well as mutations which enhance the rate of mutational change (Stratton et al., 2009). Several types of genetic mutations have been identified in human cancers, including: subtle sequence changes (for example, base substitutions); alterations in chromosome number; chromosome translocations and gene amplifications (Lengauer et al., 1998). Clonal expansion may also be initiated by non-mutational epigenetic changes that alter gene expression and chromatin structure (Berdasco and Esteller, 2010, Hanahan and Weinberg, 2011).

To ensure that an anti-cancer strategy is selectively toxic to cancer cells, it is important to target a unique property of cancer cells which is not shared by normal cells. Cancer cells possess genetic mutations that distinguish them from

normal cells. The concept of synthetic lethality can be exploited to specifically target cancer cells that contain genetic mutations not present in normal cells. Synthetic lethality is defined as a type of genetic interaction in which the combination of mutations in two genes results in lethality, but mutation of either gene alone has no effect on cell viability. The presence of one of these genetic mutations in cancer cells, but not in normal cells, creates an opportunity to selectively kill cancer cells by mimicking the effect of the second genetic mutation using targeted therapies. Since synthetic lethal targeting will only kill cells with the specific cancer associated mutation, this therapeutic strategy would most likely have less toxic side effects compared to standard chemotherapy strategies (McLornan et al., 2014, Luo et al., 2009, Chan and Giaccia, 2011). Significant effort is currently being made to identify synthetic lethal interactions for the development of novel targeted anti-cancer therapies. Interestingly, studies have revealed that mutations in the genes BRCA1 or BRAC2, which are associated with significant risk of breast cancer, display synthetic lethality with the DNA repair enzyme poly(adenosine diphosphate [ADP]-ribose) polymerase (PARP) (Bryant et al., 2005, Farmer et al., 2005). Clinical trials have shown that treatment of patients (whose breast tumours contain mutations in BRCA1 and BRCA2) with PARP inhibitors results in an anti-tumour response with limited off-target toxicities (Fong et al., 2009, Tutt et al., 2010, McLornan et al., 2014).

Although a diverse range of genetic mutations contribute to the development and maintenance of cancer, several studies have shown that cancer cells often acquire an increased dependence on a single oncogenic signalling pathway, or oncoprotein, for their uncontrolled growth and survival. Strikingly, inactivation of the same signalling pathway, or protein, in normal cells causes minimal

damage. These experimental observations have led to the development of “molecular targeted therapies”, which aim to kill cancer cells by inhibiting the molecules selectively essential for cancer survival (Higgins and Baselga, 2011, Weinstein, 2002, Weinstein and Joe, 2006).

1.8.2 Breast cancer subtypes

Breast cancer is the most frequently diagnosed cancer in women worldwide, accounting for an estimated 23% (1.38 million) of new cancer cases each year. Although significant progress has been made in the diagnosis and treatment of breast cancer, the fact remains that it is still the leading cause of cancer death in women worldwide, accounting for an estimated 500,000 deaths worldwide each year (Jemal et al., 2010, Ferlay et al., 2010).

Gene expression studies has revealed that breast cancers can be classified into the following four main subtypes: luminal A, luminal B, HER2+ (human epidermal growth factor receptor 2) and triple negative (TN) breast cancer (also known as basal like) (Sorlie et al., 2001, Sorlie et al., 2003, Perou et al., 2000, Network, 2012). These main subtypes are heterogeneous in terms of their mutational spectrum, prevalence rates, disease progression and response to treatment (Carey et al., 2006, van 't Veer et al., 2002, Polyak and Metzger Filho, 2012). In additional to the four main subtypes, breast cancers can also be classified into several other subgroups (Bertos and Park, 2011, Network, 2012).

Luminal A and B tumours, which represent an estimated 70% of breast cancer cases, often express progesterone and estrogen receptors and generally respond well to hormone therapy such as tamoxifen. Around 25% of breast tumours display amplification/ overexpression of the RTK HER2 and these are

classified as HER2+ (Polyak and Metzger Filho, 2012) (Bertos and Park, 2011). HER2+ breast cancer patients were previously reported have a poor prognosis, but the advent of HER2-targeted therapies, such as the anti-HER2 monoclonal antibody trastuzumab, has significantly improved the prognosis of HER2+ breast cancer patients (Smith et al., 2007, Carter et al., 1992, Carey et al., 2006, Slamon et al., 1987, Sorlie et al., 2001). However, the emergence of resistance to HER2-targeted therapies is common (Arteaga et al., 2012). Triple Negative (TN) breast tumours (also known as basal like), which represent 10% of breast cancer cases, predominately lack HER2 overexpression and lack expression of the progesterone and estrogen receptors. Currently there are no effective therapies for the treatment of TN breast cancer and consequently this subtype is associated with poor clinical prognosis (Nielsen et al., 2004, Sorlie et al., 2003, Polyak and Metzger Filho, 2012, Sorlie et al., 2001).

1.8.3 Genetic mutations found in breast cancer

Comprehensive analyses of the breast cancer genome has revealed the most frequent genetic mutations found in breast cancer (Stephens et al., 2012, Banerji et al., 2012, Kan et al., 2010, Network, 2012, Polyak and Metzger Filho, 2012). This mutational information has helped identify the key oncogenic signalling pathways in breast cancer, which has revealed several new therapeutic targets (Higgins and Baselga, 2011, Polyak and Metzger Filho, 2012). A recent study by the Cancer Genome Atlas Network reported the most frequent alterations found in the four main breast cancer subtypes (Luminal A, Luminal B, HER2+ and TN) by analysing patient-derived breast tumours using exome sequencing, DNA methylation, genomic DNA copy number arrays,

messenger RNA arrays, microRNA sequencing and reverse-phase protein arrays (Network, 2012). The main findings from this study are summarised in Table 1.2. This study, as well as several others, observed that several components of the PI3K-Akt-mTOR signalling pathway (PIK3CA, PTEN, INPP4B) are frequency altered in breast tumours. In fact the PI3K pathway is the most frequently mutated pathway in breast cancer and an estimated 70% of breast tumours display hyper-activated PI3K signalling (Miller et al., 2011, Polyak and Metzger Filho, 2012, Network, 2012, Wood et al., 2007, Kan et al., 2010, Banerji et al., 2012). The impact of PI3K pathway mutations on breast cancer will be described in the introduction of chapter 5.

Gene	Protein	Alteration	Frequency
PIK3CA	PI3K p110α catalytic subunit	mutation/ activation	LuA- 49% LuB- 32% TN- 7% HER2- 42%
PTEN	PTEN	mutation/deletion loss of expression	LuA- 13% LuB- 24% TN- 35% HER2- 19%
INPP4B	INPP4B	loss	LuA- 9% LuB- 16% TN- 30% HER2- 30%
TP53	p53	mutation/ inactivation	LuA- 12% LuB- 32% TN- 84% HER2- 75%
MDM2	E3 ubiquitin-protein ligase Mdm2	gain	LuA- 14% LuB- 31% TN- 14% HER2- 30%
CCND1	Cyclin D1	amplification	LuA- 29% LuB- 58% HER2- 38%
CDK4	CDK4	amplification	LuA- 14% LuB- 25% HER2- 24%
GATA3	GATA3	mutation	LuA- 14% LuB- 15% TN- 2% HER2- 2%
MAP3K1	MAP3K1	mutation	LuA- 14% LuB- 5% HER2- 2%
MAP2K4	MAP2K4	mutation	LuA- 7% LuB- 2% HER2- 2%
MYC	c-Myc	amplification	TN- 40%

Table 1.2 Most common genetic alterations found in human breast tumours

LuA= luminal A, LuB= luminal B, TN=triple negative, HER2= HER2 amplified

Adapted from (Network, 2012).

1.9 Deregulation of mRNA translation in cancer

The development of cancer involves the deregulation of processes that are essential for normal cell growth. The translation of RNA into protein (mRNA translation) is a tightly regulated process that is essential for cell growth and proliferation. One of the fundamental features of cancer cells is their ability to proliferate in a deregulated manner, and the increased proliferation of cancer cells is often associated with enhanced mRNA translation, either global or gene-specific (Johnson et al., 1976, Blagden and Willis, 2011, Silvera et al., 2010).

The rate-limiting step of mRNA translation is reported to be translation initiation; in which the 43S pre-initiation complex associates with the 7-methylguanosine (methyl cap) found at the 5' end of mRNA. This association is mediated by the eIF4F complex which consists of: (1) eIF4E, an mRNA 5' methyl cap binding protein, (2) eIF4G, a scaffold protein and (3) eIF4A, an ATP-dependent RNA helicase. Oncogenic signalling pathways stimulate mRNA translation through the regulation of eIF4F complex assembly and components of the eIF4F complex can become oncogenic when overexpressed. The deregulation of the eIF4F complex in cancer will be described in this subchapter (Blagden and Willis, 2011, Silvera et al., 2010, Gingras et al., 1999, Sonenberg and Hinnebusch, 2009).

1.9.1 eIF4E

eIF4E specifically binds to the mRNA methyl cap, thereby facilitating the recruitment of the 43S pre-initiation complex to the 5' end of mRNA. The binding of eIF4E to the methyl cap is essential for mRNA translation. There is considerable evidence that expression of eIF4E is rate-limiting for eIF4F

complex formation and cap-dependent mRNA translation (De Benedetti et al., 1991, Duncan et al., 1987, Hiremath et al., 1985). A numbers of studies have reported eIF4E to be oncogenic. Exogenous overexpression of eIF4E enhances cell proliferation and leads to cell transformation (Lazaris-Karatzas et al., 1990, De Benedetti and Rhoads, 1990, De Benedetti and Graff, 2004, Avdulov et al., 2004). Conversely, depletion of eIF4E expression from cells impairs cell proliferation and reverses the eIF4E-mediated oncogenic phenotype (Soni et al., 2008). Moreover, mouse studies have observed that eIF4E overexpression is sufficient to induce tumorigenesis (Ruggero et al., 2004, Wendel et al., 2004b). Data has demonstrated that the ability of eIF4E to stimulate mRNA translation is required for eIF4E-mediated oncogenesis. For example, overexpression of an eIF4E mutant which cannot bind the 5' methyl cap is unable to promote lymphomagenesis in mice (Ruggero et al., 2004) and a reduction in the affinity of eIF4E for the methyl cap suppresses oncogenic transformation (Cohen et al., 2001). Moreover, overexpression of 4E-BP1, which inhibits eIF4E assembly into the eIF4F complex, reverses the eIF4E-induced transformed phenotype in mammary epithelial cells and rodent fibroblasts (Rousseau et al., 1996a, Avdulov et al., 2004) and reduces the tumorigenicty of human breast cancer cells injected into nude mice (Avdulov et al., 2004). Data from several studies has revealed that eIF4E promotes cell proliferation and oncogenesis by enhancing the translation of several mRNAs which encode for proteins which stimulates cell growth (Koromilas et al., 1992, De Benedetti and Harris, 1999, Rosenwald et al., 1993, Shantz and Pegg, 1994, Kevil et al., 1995). The full spectrum of these "eIF4E-dependent" mRNAs remains unknown, but mRNAs with complex secondary structures in their 5' UTRs have been shown to be strongly dependent on eIF4E expression for their

translation (Graff et al., 2007, Koromilas et al., 1992, Kevil et al., 1995, Rousseau et al., 1996b). (De Benedetti and Harris, 1999). Moreover, overexpression of eIF4E enhances the nuclear export of specific mRNAs and this is known to be required for eIF4E-mediated oncogenesis (Rousseau et al., 1996b, Culjkovic et al., 2005, Cohen et al., 2001, Strudwick and Borden, 2002). eIF4E expression is elevated in several types of human cancers such as: breast, head and neck, lung and prostate cancer (Li et al., 1998, De Benedetti and Harris, 1999, DeFatta et al., 1999, Nathan et al., 2004, Wang et al., 2009, Coleman et al., 2009). Since eIF4E overexpression in breast cancer correlates with poor clinical outcome and reduced patient survival it is likely that eIF4E contributes to human breast cancer (De Benedetti and Harris, 1999, Li et al., 1997, Li et al., 1998, Scott et al., 1998, Kerekatte et al., 1995, Coleman et al., 2009). However, eIF4E expression is not always found to be elevated in breast cancer (Avdulov et al., 2004). Several strategies to target eIF4E expression/ activity in human cancer are currently being explored and these will be described in section 1.10.

1.9.2 eIF4G

eIF4G acts as a scaffold protein and facilitates the recruitment of the 43S pre-initiation complex to the mRNA via interactions with eIF3 and eIF4E (Gingras et al., 1999, Sonenberg and Hinnebusch, 2009). Although not as well studied as eIF4E-mediated oncogenesis, eIF4G has been reported to be oncogenic when overexpressed. Exogenous overexpression of eIF4G in NIH3T3 cells leads to anchorage independent growth and these cells form tumours when injected in nude mice (Fukuchi-Shimogori et al., 1997). Moreover, elevated expression of

eIF4G enhances tumour formation in a xenograft model of inflammatory breast cancer (Silvera et al., 2009). The majority of breast tumours derived from patients with advance stage breast cancer, or inflammatory breast cancer, exhibit elevated eIF4G expression (Braunstein et al., 2007, Silvera et al., 2009).The reported overexpression of eIF4G in a subset of human cancers provides evidence to support an oncogenic role for eIF4G in human cancer (Braunstein et al., 2007, Silvera et al., 2009, Bauer et al., 2001). The interaction between eIF4E and eIF4G has been targeted for anti-cancer therapeutics and this is described in section 1.10.2.

1.9.3 eIF4A

eIF4A, an ATP dependent RNA helicase, unwinds the secondary structure in the 5' UTR of mRNA allowing the efficient recruitment of the 43S pre-initiation complex to the mRNA (Rogers et al., 1999). An oncogenic role for eIF4A has not yet been established (Malina et al., 2012, Silvera et al., 2010), but eIF4A mRNA has been shown to be elevated in human melanoma cells and primary human hepatocellular carcinomas (Eberle et al., 1997, Shuda et al., 2000). Despite eIF4A having no established role in oncogenesis, inhibitors of eIF4A have potent anti-cancer activity and are currently in clinical development (section 1.10.4).

1.9.4 PI3K-Akt-mTOR signalling

Most human cancers are caused by the hyper-activation of signalling pathways which regulate processes essential for cell growth, such as protein synthesis. The PI3K-Akt-mTOR signalling pathway is one of the most frequently mutated pathways in human cancers. A major downstream target of PI3K is the serine/threonine kinase mammalian target of rapamycin (mTOR) (Blagden and Willis, 2011, Silvera et al., 2010, Proud, 2007, Rajasekhar et al., 2003). mTOR, which is the target of the macrolide rapamycin, regulates cell growth and protein synthesis in response to cellular nutrient and energy levels (Laplante and Sabatini, 2012, Sabatini et al., 1994, Sabers et al., 1995). mTOR forms two multisubunit complexes : mTORC1 and mTORC2. mTORC1 is known to be extremely sensitive to inhibition by rapamycin, whereas mTORC2 is only sensitive to chronic rapamycin treatment. mTORC1 can stimulate cap-dependent translation via the regulation of eIF4F complex formation (Laplante and Sabatini, 2012, Ma and Blenis, 2009).

The regulation of cap-dependent mRNA translation by the PI3K-Akt-mTOR signalling pathway is shown in Figure 1.9. The ability of the PI3K pathway to stimulate mRNA translation via the activation of mTORC1 is essential for its oncogenic capabilities (Hsieh et al., 2010, Skeen et al., 2006). The activation of RTKs by mitogenic signals (growth factors, cytokines, hormones) stimulates PI3K, which in turn, phosphorylates phosphatidylinositol 4,5 bisphosphate ($PI(4,5)P_2$) to produce phosphatidylinositol 3,4,5- trisphosphate ($PI(3,4,5)P_3$). The catalytic activity of PI3K is antagonised by the tumour suppressor phosphatase and tensin homolog (PTEN), which dephosphorylates $PI(3,4,5)P_3$ to produce $PI(4,5)P_2$ (Myers et al., 1998, Maehama and Dixon, 1998). $PI(3,4,5)P_3$ production leads to Akt recruitment and activation (Manning and

Cantley, 2007, Liu et al., 2009b). Activated Akt stimulates mTORC1 by two mechanisms: (1) inhibiting PRAS40, a negative regulator inhibitor of mTORC1 activity (2) phosphorylating and inactivating the tuberous sclerosis complex (TSC) complex (Inoki et al., 2002, Kovacina et al., 2003, Sancak et al., 2007). The TSC complex, which consists of TSC1 and TSC2, functions as a GTPase-activating protein, inhibiting the small G-protein Rheb. Inactivation of the TSC2 complex by Akt-mediated phosphorylation allows Rheb-GTP activation, which in turn, activates mTORC1 (Inoki et al., 2002, Inoki et al., 2003a, Long et al., 2005). AMP-activated protein kinase (AMPK) is an important energy and nutrient sensor kinase (Hardie et al., 2012). Activated AMPK is able to inhibit mTORC1 by phosphorylating and activating the TSC2 complex (Inoki et al., 2003b). AMPK activity is regulated by the tumour suppressor kinase LKB1 (Liver Kinase B1), which is frequently inactivated in cancer resulting in enhanced mTORC1 activity (Shaw et al., 2004, Woods et al., 2003). mTORC1 signalling drives several cellular processes that regulate protein synthesis, such as mRNA translation and ribosome biogenesis (Guertin and Sabatini, 2007). Activated mTORC1 enhances cap-dependent translation by phosphorylating its downstream targets 4E-BPs and S6K (Pearson et al., 1995, Beretta et al., 1996, Gingras et al., 1998). The 4E-BPs bind to eIF4E, the methyl cap binding protein, and inhibit eIF4F complex formation by preventing eIF4E interacting with the eIF4G scaffold protein (Mader et al., 1995, Marcotrigiano et al., 1999, Haghighat et al., 1995). Phosphorylated 4E-BP is no longer able to interact with eIF4E, thereby permitting eIF4F complex formation and allowing cap dependent translation to proceed (Pause et al., 1994, Brunn et al., 1997, Gingras et al., 1999). eIF4E-mediated mRNA translation is proposed to be one of the main

effector pathways that drives cell proliferation downstream of mTORC1 (Laplante and Sabatini, 2013, Laplante and Sabatini, 2012, Populo et al., 2012).

Activated mTORC1 also phosphorylates and activates S6K. Activated S6K then enhances cap-dependent translation by phosphorylating its downstream substrates, such as S6 ribosomal protein and eIF4B. The exact mechanisms by which these S6K-activated substrates mediate their effects on translation remain unresolved. However, studies have reported that the S6K-phosphorylation of eIF4B enhances translation possibility by stimulating eIF4A helicase activity (Holz et al., 2005, Shahbazian et al., 2006). Furthermore, activated S6 kinase phosphorylates the CBP80 subunit of CBC, which enhances the affinity of the CBC for the 7-methylguanosine cap (Wilson et al., 2000). As described above, the CBC promotes several steps in gene expression and mediates several functions of the methyl cap (Figure 1.7) (Ma et al., 2008). It is proposed that S6K-mediated phosphorylation of CBC stimulates mRNA translation, but the precise mechanisms of this remains to be determined.

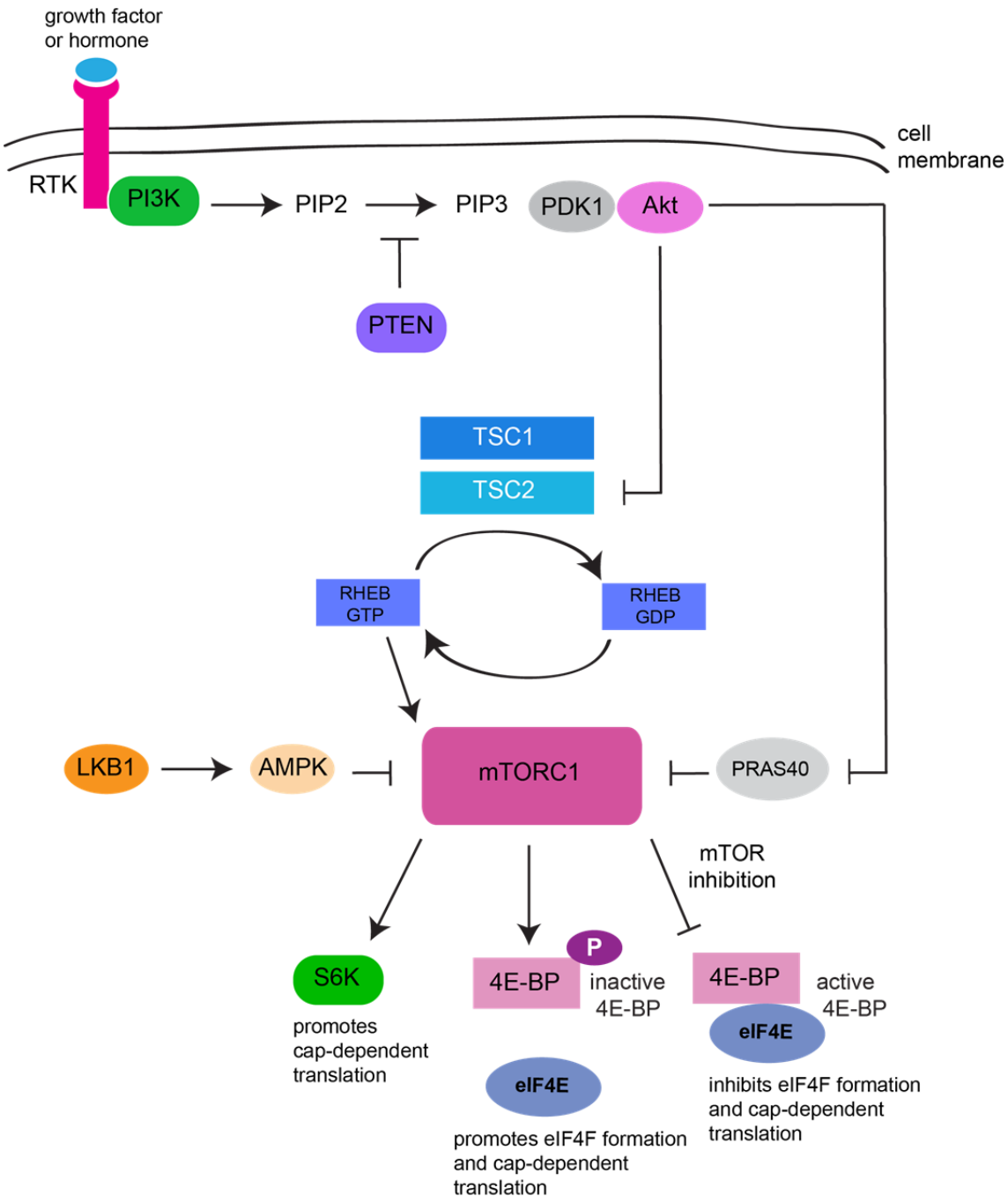


Figure 1.9 The regulation of cap-dependent translation by the PI3K-Akt-mTOR signalling pathway

PI3K signalling stimulates cap-dependent translation via mTORC1 activation, as described in more detail in the text. Adapted from (Thomas, 2006, Silvera et al., 2010, Laplante and Sabatini, 2012).

1.10 Therapeutic strategies to inhibit mRNA translation

As discussed above, deregulated mRNA translation is associated with cancer. The assembly of the eIF4F complex is regulated by oncogenic pathways and components of the complex can become oncogenic. Therefore, there has been considerable effort into developing strategies to inhibit mRNA translation for cancer therapeutics.

Most of the components of the eIF4F complex lack enzymatic activity, which has hindered the development of small molecule inhibitors against the complex. Nonetheless, a number of strategies have been developed to target the eIF4F complex and these are illustrated in Figure 1.10. These strategies include: (1) inhibition of eIF4E expression/activity, (2) blocking the interaction between eIF4E-eIF4G, (3) blocking the interaction between eIF4E and the mRNA 5' methyl cap, (4) inhibition of eIF4A activity, or (5) inhibition of mTOR signalling (Silvera et al., 2010, Blagden and Willis, 2011, Malina et al., 2012).

Although not exhaustive, strategies to therapeutically target cap-dependent mRNA translation in clinical development are summarised in Table 1.3.

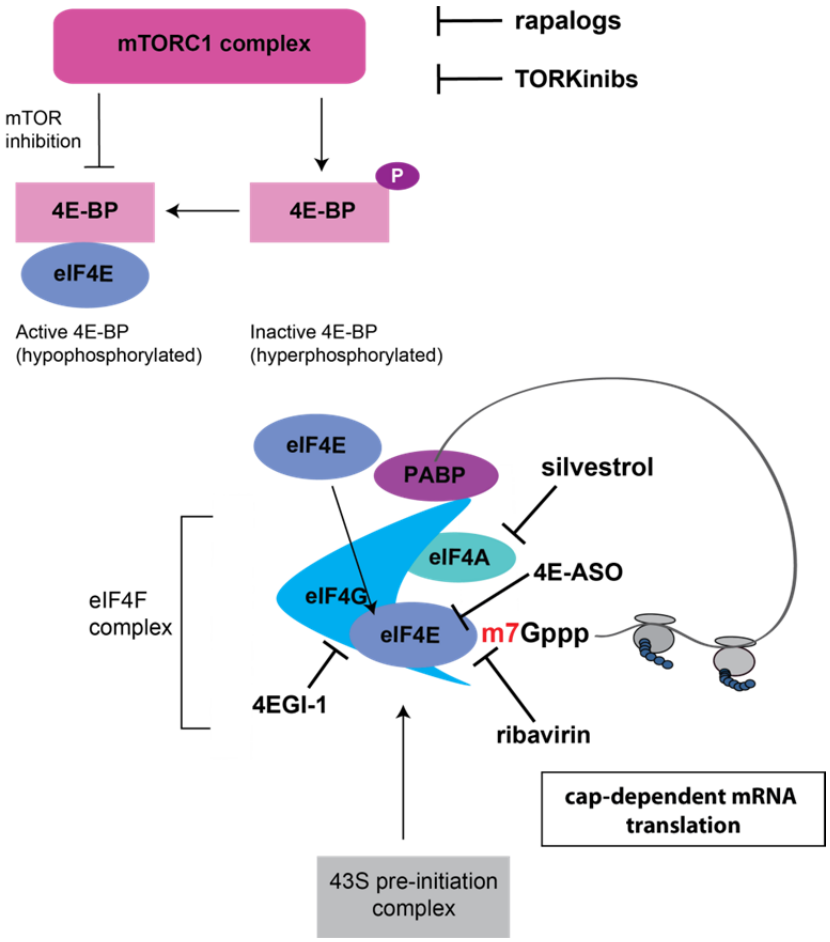


Figure 1.10 Targeting cap-dependent mRNA translation in cancer

Active mTORC1 stimulates cap-dependent translation by phosphorylating the 4E-BPs, which allows eIF4E to bind eIF4G and facilitates eIF4F complex (eIF4A, eIF4E and eIF4G) formation. Binding of eIF4E to the 5' mRNA methyl cap is essential for recruiting the eIF4F complex to the mRNA and this interaction is required for cap-dependent translation. PABP binds the 3' end of mRNA and eIF4G leading to mRNA circulation. The eIF4F complex leads to the recruitment of the 43S pre-initiation (consisting of 40S ribosomal subunit, eIF2-GTP-Met tRNA ternary complex, eIF3, eIF1, eIF1A and most likely eIF5) to the 5' of mRNA. The 43S pre-initiation complex scans the 5'UTR until it finds the initiation codon. Following the dissociation of several translation factors, the 60S ribosome joins to form the 80S translation initiation complex and cap-dependent mRNA translation initiates. Although not exhaustive, strategies to target cap-dependent mRNA translation in cancer include directly targeting eIF4F complex components (4EGI-1, ribavirin, 4E-ASO and silvestrol) or inhibiting mTORC1 activity (rapalogs and TORKinibs).

Target	Agent	Mode of action	Stage of development	Results
eIF4E	4E-ASO	antisense oligonucleotide	preclinical animal	- reduction in tumour growth/ angiogenesis
eIF4E	4E-ASO	antisense oligonucleotide	phase I clinical	- no tumour reduction and minimal toxicities - phase I with combination chemotherapy being tested
eIF4G-eIF4E interaction	4EGI-1	blocks eIF4G-eIF4E interaction	preclinical	- selectively toxic to cancer cells
eIF4E-methyl cap	ribavirin	blocks eIF4E-methyl cap interaction (controversial)	phase I clinical	- significant single agent activity in patients with AML - tumour reduction in patients with elevated eIF4E expression - minimal toxicities
eIF4A	silvestrol, botanical molecule	inhibits eIF4A activity	preclinical animal	- reduction in tumour growth and minimal toxcities
mTORC1	rapalogs: temisirolimus everolimus	inhibits mTORC1 activity	approved, phase I, II, III	- approved for certain cancers - resistance to treatment frequent - clinical trials: other cancers and combination therapies in progress
mTORC1 + mTORC2	AZD8055 AZD2014	inhibits both mTORC1 and mTORC2 activity	phase I, II	- preclinical trials produced promising results - clinical trial: advanced solid tumours in progress

Table 1.3 The strategies to therapeutically target mRNA translation in preclinical or clinical development

Adapted from (Blagden and Willis, 2011, Malina et al., 2012, Wander et al., 2011) and data obtained from http://www.clinicaltrials.gov, accessed August 2014.

1.10.1 Inhibition of eIF4E expression

eIF4E expression has been targeted using the antisense oligonucleotides 4E-ASO, which inhibits eIF4E expression by binding the eIF4E gene. In breast and prostate xenograft models, treatment of 4E-ASO reduces tumour size while causing limited toxicity to normal tissue (Graff et al., 2007). This study demonstrated for the first time that tumour cells exhibit an enhanced dependency on eIF4E expression for survival, in comparison to normal cells. However, a phase II clinical trial testing the efficacy of 4E-ASO in patients with advanced cancer showed that despite an effective reduction in eIF4E expression, no significant changes in tumour size were observed (Hong et al., 2011). Potential explanations for this include: (1) eIF4E does not function as an oncoprotein in these tumours and (2) the depletion of eIF4E expression was not sufficient enough to exert a potent anti-tumour effect.

1.10.2 Blocking the eIF4E-eIF4G interaction

The Wagner group identified a small molecule called 4EGI-1, that inhibits cap-dependent translation by blocking the eIF4E-eIF4G interaction. Treatment of cells with 4EGI-1 preferentially reduced the expression of eIF4E-dependent oncogenic proteins, and inhibited the proliferation of lung cancer cell lines. Furthermore, 4EGI-1 was demonstrated to be selectivity toxic to transformed cells compared to non-transformed cells (Moerke et al., 2007). Although the results from this study indicated that 4EGI-1 is not potent enough for use as a single agent, the use of this drug as part of combination therapies is currently being explored for the treatment of cancer (Fan et al., 2010).

1.10.3 Blocking the eIF4E- 5' mRNA methyl cap interaction

Since the interaction between eIF4E and the 5' methyl cap is essential for mRNA translation and eIF4E-mediated oncogenesis (Ruggero et al., 2004), it is likely that targeting the eIF4E-5' methyl cap interaction is an effective anti-proliferative strategy. The drug ribavirin, which is approved for the treatment of hepatitis C, is structurally similar to the mRNA 5' methyl cap and it has been reported that ribavirin sequesters eIF4E away from the mRNA methyl cap (Malina et al., 2012). Strikingly, treatment with ribavirin was found to selectively inhibit the translation of eIF4E-dependent mRNAs and inhibit tumour growth in mice (Kentsis et al., 2004). A phase I clinical trial assessing the effect of ribavirin on patients with acute myeloid leukemia demonstrated that ribavirin treatment lead to significant reduction in eIF4E overexpressing tumours while causing limited toxic side effects (Assouline et al., 2009). This study provided the first clinical evidence that targeting the eIF4E-mRNA methyl cap interaction is an effective anti-cancer strategy in eIF4E overexpressing tumours. However, since two additional studies have been unable to detect an interaction between ribavirin and recombinant eIF4E, it remains controversial whether ribavirin inhibits the eIF4E-methyl cap interaction (Yan et al., 2005, Westman et al., 2005).

1.10.4 Inhibition of eIF4A

An oncogenic role for eIF4A has not yet been established. However, eIF4A inhibitors derived from nature such as paleamine A, hippuristanol and silvestrol exert powerful anti-proliferative effects and are therefore being investigated as therapeutic anti-cancer inhibitors (Malina et al., 2012, Blagden and Willis, 2011).

Silvestrol, which acts by removing eIF4A from the eIF4F complex, is the most clinically developed eIF4A inhibitor. In various cancer cell lines, as well as in mouse tumour xenograft models, treatment with silvestrol exerts anti-tumour activity (Cencic et al., 2009, Cencic et al., 2010). Preclinical studies have shown that silvestrol is particularly effective against B-cell leukemias and it is currently in clinical development for use against aggressive leukemias and lymphomas (Lucas et al., 2009, Lucas et al., 2010).

1.10.5 Inhibition of mTOR signalling

As discussed above, the PI3K-Akt-mTOR pathway stimulates mRNA translation via the regulation of mTORC1. PI3K-Akt-mTOR signalling is frequently hyper-activated in cancer leading to deregulated protein synthesis. Thus, therapeutically targeting this pathway is an attractive anti-cancer strategy. Although inhibitors against several components of the PI3K pathway are in clinical development for the treatment of cancer, only mTOR inhibitors have been approved for use in the clinic (Silvera et al., 2010, Blagden and Willis, 2011). Rapamycin and its derivatives, known as rapalogs, suppress mRNA translation by inhibiting mTORC1 and exert anti-tumour effects (Silvera et al., 2010, Pedersen et al., 1997, Shor et al., 2008).The rapalogs temsirolimus, and everolimus are approved for the treatment of metastatic renal cell carcinoma but the anti-tumour effect of these drugs is often short term and resistance is frequent. The rapalogs are currently being explored in numerous clinical trials for use against several types of human cancers (Malina et al., 2012, Blagden and Willis, 2011, Meric-Bernstam and Gonzalez-Angulo, 2009). Data from clinical trials have shown that rapalogs are only effective against a subset of

human cancers. For example, rapalogs are effective as single therapy agents in patients with metastatic endometrial cancer and mantle cell lymphoma (Hess et al., 2009, Oza et al., 2008), but they have limited single agent activity in patients with metastatic breast cancer or small-cell lung cancer (Pandya et al., 2007, Chan et al., 2005). The long term clinical response to rapalogs is known to be limited by the activation of feedback mechanisms, as well as the activation of redundant signalling pathways (Carew et al., 2011). This may be because rapamycin does not fully de-phosphorylate the 4E-BPs, thereby allowing cap-dependent translation to persist in response to treatment (Choo et al., 2008, Thoreen et al., 2009). The fact that eIF4E expression confers resistance to mTOR inhibitors suggests that the combined inhibition of eIF4E expression/ activity with mTOR inhibitor treatment may synergise to reduce resistance (Wendel et al., 2004b, Cope et al., 2013, Sun et al., 2005).

The activity of mTORC2 is only sensitive to chronic rapamycin treatment and resistance to rapalogs can occur by mTORC2-mediated feedback activation of the PI3K signalling pathway. These observations have led to the development of second generation mTOR kinase inhibitors (TORKinibs), which inhibit the catalytic activity of both mTORC1 and mTORC2. Several of these are currently in either preclinical, or clinical, development (Table 1.3) (Blagden and Willis, 2011, Benjamin et al., 2011). TORKinibs have been shown to be superior to rapalogs in their ability to inhibit mTORC1-mediated 4E-BP phosphorylation and suppress cell growth and protein synthesis (Feldman et al., 2009, Thoreen et al., 2009).

As well as mTOR inhibitors, several inhibitors against PI3K and Akt are in clinical trials and have shown significant anti-tumour effects (Liu et al., 2009a,

Pal and Mandal, 2012). The therapeutic targeting of PI3K in cancer will be described in more detail in the introduction of chapter 5.

1.11 mRNA cap methylation and cancer

There is considerable evidence that eIF4E is oncogenic and eIF4E expression is often found to be elevated in several types of human cancers, including breast cancer (De Benedetti and Graff, 2004, De Benedetti and Harris, 1999, Gingras et al., 1999). The binding of eIF4E to the methyl cap has been reported to be essential for the translation of most mRNAs and is important for eIF4E-mediated oncogenesis. These observations suggest that mRNA cap methylation has the potential to contribute to cancer (Ruggero et al., 2004, Cohen et al., 2001). Indeed, emerging evidence has shown that the mRNA methyl cap is associated with oncogenic transformation. For example, exogenous overexpression of RNMT in mammary epithelial cells promotes cell transformation (Cowling, 2009a). Moreover, the oncoprotein c-Myc upregulates cap methylation and this is essential for Myc-driven protein synthesis, cell transformation and cell proliferation (Fernandez-Sanchez et al., 2009a). Strikingly, inhibition of cap methylation is reported to be synthetic lethal with elevated c-Myc expression. The above observations lead us to the intriguing possibility that inhibition of cap methylation may be an effective anti-cancer strategy. Since the methyl cap is essential for the translation of most mRNAs, the inhibition of cap methylation has the potential to inhibit the action of any oncogene that deregulates gene expression. Thus, targeting RNMT would likely have wide efficacy and could be effective in tumours driven by different oncogenes. All of the above experiments were carried out in non-transformed

experimental cell lines and it is important to establish whether inhibition of cap methylation selectively supresses the proliferation of cancer cells.

1.12 Summary and aims of this PhD thesis:

Breast cancer is the leading cause of cancer death in women worldwide. Significant advances have been made in the treatment of breast cancer, but new therapeutic approaches are required. Protein synthesis is often found to be deregulated in cancer and there has been significant effort into developing strategies to inhibit protein synthesis for anti-cancer therapeutics. The 7-methylguanosine cap structure found at the 5' end of mRNA is essential for mRNA translation and it has been shown to promote several steps in gene expression. In humans, RNMT catalyses the methylation of the guanosine cap structure and RNMT expression is essential for mRNA cap methylation and efficient gene expression. Emerging evidence suggests that inhibition of cap methylation may be an effective therapeutic anti-cancer strategy. The aim of this thesis is to assess whether depletion of RNMT selectively inhibits the proliferation of breast cancer cells.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Laboratory equipment

Autoradiography cassette (Siemens), Automatic film processor (Konica Corporation), C1000 thermal cycler (Bio-Rad), Cell countess (Invitrogen), Centrifuge 5415-R (Eppendorf), CO_2 incubators (Mackay + Lynn), Mini-PROTEAN Tetra Cell (Bio-Rad), Mini-Sub Cell GT Cell (Bio-Rad), Mini-Trans-Blot Cell (Bio-Rad), Nanodrop Spectrometer 1000 (Thermo Scientific), Phosphorimager FLA-500 (FujiFilm), Phosphor screen (FujiFilm), Pipettes (Greiner), Tissue culture safety cabinets (Medical Air technology), Versa Max microplate reader (Molecular Devices), X-Cell SureLock Mini-Cell (Life Technologies).

2.1.2 Tissue culture reagents

Antibiotic – Antimycotic (Anti-Anti) (Gibco, Life Technologies), Cryovials (Corning), Dulbecco's phosphate buffered saline (DPBS) (Gibco, Life Technologies), Dimethyl sulfoxide (DMSO) (Sigma-aldrich), Dulbecco's modified eagle medium nutrient mixture F-12 ham (Gibco, Life Technologies), Epidermal growth factor (EGF) (Sigma-aldrich), G-418 (ForMedium), HyClone fetal bovine serum (FBS) (Thermo Scientific), Insulin solution human (Sigma-aldrich), L-Glutamine (Gibco, Life Technologies), Lipofectamine 2000 transfection reagent (Life Technologies), Lipofectamine RNAiMax transfection reagent (Life Technologies), Nunc 25 cm^2 and 75 cm^2 flasks (Thermo

Scientific), NuPAGE (Novex) 4-12% Bis-Tris protein gels (Life Technologies), Polybrene (Sigma-aldrich), Petri dishes (Corning), RPMI 1640 Medium (Gibco, Life Technologies), Trypan blue solution (0.4%) (Gibco, Life Technologies), Trypsin-EDTA solution (Gibco, Life Technologies).

2.1.3 Commercial reagents

2-Mercaptoethanol (Sigma-aldrich), [35S] Protein labelling mix (2 mCi) (Perkin Elmer), 40% Acrylamide : Bis-acrylamide 29:1 (Flowgen Biosciences), 96-well hard-shell PCR frame Plate (Bio-Rad), [α-32P] guanonsine-5' triphosphate (GTP) (500 μCi) (Perkin Elmer), Acetic acid (VWR International), Agarose (Life technologies), Ammonium sulphate (Sigma-aldrich), Aprotinin (Sigma-aldrich), BenchMark pre-stained protein ladder (Life technologies), Bio-Rad protein assay (Bio-Rad), Bovine serum assay (Bio-Rad), Bromophenol blue (Sigma-aldrich), Dithiothreitol (DTT) (ForMedium), Dried skimmed milk (Marvel), Ethanol (VWR International), Ethylenediamine tetraacetic acid (EDTA) (Sigma-aldrich), Ethylene glycol tetraacetic acid (EGTA) (Sigma-aldrich), GeneJET RNA Purification kit (Thermo Scientific), Glycerol (VWR International), Hydrochloric acid (VWR International), Immobilon transfer membrane (Millipore), iScript cDNA synthesis kit (Bio-Rad), Leupeptin hydrochloride (Sigma-aldrich), Magnesium chloride ($MgCl_2$) (Sigma-aldrich), Methanol (VWR International), Microseal adhesive seals (BioRad), *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine (TEMED) (VWR International), NuPAGE® Novex® 4-12% Bis-Tris protein gels (Life Technologies), NuPAGE® Novex® MES SDS running buffer (Life Technologies), NuPAGE® Novex® MOPS SDS running buffer (Life Technologies), P1 nuclease (Sigma-aldrich), PCR tubes (Axygen),

Pepstatin (Sigma-aldrich), Phosphatase inhibitor cocktail 2 (Sigma-aldrich), Phosphatase inhibitor cocktail 3 (Sigma-aldrich), Phenol:Chloroform: isoamylalcohol (Ambion), Plasmid maxi kit (Qiagen), Potassium chloride (KCl) (Sigma-aldrich), QIAquick PCR purification kit (Qiagen), Recombinant RNasin ribonuclease inhibitor (rRNasin) (Promega), S-adenosyl-methionine (SAM) (Sigma-aldrich), SimplyBlue safe stain (Life technologies), Sodium acetate (Ambicon), Sodium chloride (NaCl) (VWR International), Sodium dodecyl sulfate (SDS) (Sigma), Sodium fluoride (NaF) (Sigma-aldrich), Sodium orthovanadate (Sigma-aldrich), Sodium pyrophosphate tetrabasic (Sigma-aldrich), Super signal west pico chemiluminescent substrate (Thermo Scientific), SYBR® Green FastMix ™ (Quanta Biosciences), T7 RNA polymerase (Promega), TLC PEI cellulose sheets (Merck Millipore), Tris-Base (VWR International), Triton X-100 (Sigma-aldrich), tRNA (Sigma-aldrich), Tween-20 (VWR International), X-ray films (Konica Minolta), Zinc chloride ($ZnCl_2$) (Sigma-aldrich).

2.1.4 In-house regents

LB agar plates (+ Ampicillin) and LB broth (+ Ampicillin) (Media Kitchen facility, College of Life Sciences, University of Dundee). GDC-0941, an inhibitor of class I PI3Ks (Folkes et al., 2008b), and Staurosporine (a broad spectrum kinase inhibitor) were produced by the Division of Signal Transduction Therapy (College of Life Sciences, University of Dundee).

2.1.5 Antibodies

All commercial antibodies used in this thesis are shown in Table 2.1 and were used at a 1:1000 dilution in 3% BSA in TBS-Tween. All in-house antibodies used were made by DSTT and were affinity purified on CH-Sepharose covalently coupled to the antigen. The details of the in-house antibodies used in this thesis are shown in Table 2.2. The working concentration and dilution conditions used for the in-house antibodies are shown in Table 2.3.

Antibody	Supplier	Catalogue Number	Host
β- Actin	Abcam	8226	mouse
β-Tubulin	Santa Cruz Biotechnology	sc-9104	rabbit
cleaved-PARP	cell signaling	9541	rabbit
P-P70 S6 kinase Thr 389	cell signaling	9205	rabbit
P 4E-BP1 (53H11)	cell signaling	9644	rabbit
P 4E-BP1 Thr 37/46	cell signaling	2855	rabbit
P 4E-BP1 Thr 70	cell signaling	9455	rabbit
P-Akt Thr 308	cell signaling	9275	rabbit
P-Akt Ser 473	cell signaling	9271	rabbit

Table 2.1 Details of the commercial antibodies used in this thesis

Antibody	Host	Antigen	Details
9E10	mouse	Myc tag- EQKLISEEDL	DSTT
Akt	sheep	Full length human Akt (His)	DSTT, S742B, 3rd bleed
c-Myc	sheep	c-MYC (GST) (2-439)	DSTT, S996A, 3rd bleed
P70 S6 K1	sheep	Human P70 S6 K1 (25-44) AGVFDIDLDQPEDAGSEDEL	DSTT, S417B, 2nd bleed
RAM	sheep	Full length RAM (GST)	DSTT, S554D, 4th bleed
RNMT	sheep	Full length RNMT (GST)	DSTT, S523C, 3rd bleed

Table 2.2 Details of the in-house antibodies used in this thesis

The host and antigen used to raise the antibodies and the antibody reference details are indicated.

Antibody	Concentration µg/ml	Diluted in
9E10	1	3% BSA in TBS-Tween
Akt	1	5% milk in TBS-Tween
c-Myc	1	3% BSA in TBS-Tween
P70 S6 kinase	1	5% milk in TBS-Tween
RAM	0.8	3% BSA in TBS-Tween
RNMT	0.3	5% milk in TBS-Tween

Table 2.3 Working concentrations and dilution conditions used for all the in-house antibodies employed in this thesis

2.1.6 Buffers and solutions

F buffer (pH 7.05): 10 mM Tris (pH 7.05), 50 mM NaCl, 30 mM Na pyrophosphate, 50 mM NaF, 5 µM $ZnCl_2$, 10% Glycerol, 0.5% Triton X-100, 1 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, (further supplemented with 0.1 TIU (trypsin inhibitor unit) aprotinin, 1 µM pepstatin, 10 µM leupeptin and 1 mM DTT immediately before use)

SDS running buffer: 25 mM Tris, 250 mM glycine, 0.1% SDS

Transfer buffer: 25 mM Tris, 192 mM glycine, 20% methanol

TBS-Tween (pH 8.1): 25 mM Tris, 155 mM NaCl, 0.1% Tween-20

Laemmli buffer (4x): 240 mM Tris (pH 6.8), 8% SDS, 40% glycerol, bromophenol blue (further supplemented with 10% DTT immediately before use)

Methyltransferase assay buffer: 100 mM Tris (pH 8), 6 mM KCl, 1.25 mM $MgCl_2$

RNGTT buffer: 50 mM Tris (pH 7.5), 5.5 mM DTT, 1.25 mM $MgCl_2$

2.2 Methods

2.2.1 Transformation of *E.coli* and plasmid purification

For each transformation, 30 µl of competent *E.coli* cells were mixed with 2 µg of plasmid DNA and incubated on ice for 20 min. To facilitate the uptake of DNA, cells were subject to heat-shock by incubating at 45˚C for 1 min followed by a 2 min incubation on ice. For selection, cells were streaked on LB/ Amp agar plates and inverted plates were incubated overnight at 37˚C. A single isolated colony was then picked and incubated with 200 ml of LB (Amp) at 37˚C overnight, following which cells were pelleted by centrifugation at 3000 rpm for 10 min. Plasmid DNA was purified using the Qiagen plasmid Maxi kit according to manufacturer's instructions.

2.2.2 DNA sequencing

DNA sequencing was performed by DNA Sequencing & Services (MRCPPU, College of Life Sciences, University of Dundee, Scotland, www.dnaseq.co.uk) using Applied Biosystems Big-Dye Ver 3.1 chemistry on an Applied Biosystems model 3730 automated capillary DNA sequencer.

2.2.3 Cloning

All constructs used in this thesis were cloned by Dr Simone Weidlich from the Division of Signal Transduction Therapy (DSTT) cloning team. All constructs employed in this thesis are shown in Table 2.4 and encode the human version of the gene. All constructs were used to retrovirally transduce cells to produce cell lines which stably express the protein encoded in the construct.

Construct	vector	clone number
RNMT WBL-GFP	pBMN-IRES-NEO (INI)	DU42837
PI3KCA WT-Myc	pBMN-IRES-NEO (INI)	DU42773
PI3KCA C420R-Myc	pBMN-IRES-NEO (INI)	DU428802
PI3KCA E545K-Myc	pBMN-IRES-NEO (INI)	DU42774
PI3KCA H1047R-Myc	pBMN-IRES-NEO (INI)	DU42778

Table 2.4 All constructs employed in this thesis

2.2.4 DNA concentration determination

Concentration of DNA (resuspended in distilled water) was determined using a Nanodrop Spectrophotometer 1000, which was blanked using distilled water. The absorbance was measured at 260/280 nm according to the manufacturer's instructions.

2.2.5 Cell culture and maintenance

Cells were grown in 75 cm^2 flasks and maintained at 37 ˚C in a humidified incubator with 5% CO_2. The cell lines and culture media used in this thesis are shown in Table 2.5. The breast cancer cell panel was purchased directly from the American Type Culture Collection (ATCC) and the key features (Subtype, source, clinical features) of the cell lines are shown in Table 2.6. The IMEC cell line was kindly provided to me by Dr James DiRenzo (Dartmouth Medical School). When the cells were passaged, each flask of cells was washed once with PBS and then 1 ml of Trypsin was added to the cells for 5 min to dissociate them from the flask. Cells were then resuspended in fresh culture media (Table

2.5) to inactivate trypsin and 1/5th of the cell suspension was seeded into a new flask. Trypsinised immortalised mammary epithelial cells (IMEC) were resuspended in RPMI culture media supplemented with 10% FBS to inactivate trypsin and centrifuged for 3 min at 1,700 rpm. The resultant cell pellet was resuspended in DMEM F-12 Ham media (supplemented with 2 mM L-glutamine, 5 mg/ml Insulin, 10 ng/ml EGF, 0.5 µg/ml hydrocortisone and 1% Anti-Anti) and 1/5th of the cell suspension was seeded into a new flask. IMECs were used for experiments up to passage number 18 and all other cell lines were used for experiments up to passage number 30. IMEC and ZR.75.1 cells stably expressing wild-type, or activating mutants, of PI3K p110α were used for experiments up to passage number 60.

When cells were required for the immunoblot analysis of phosphorylated proteins, cells were replenished with fresh culture media 1 hr prior to cell lysis.

Cell line	Culture Media
A′	DMEM supplemented with 10% FBS, 2 mM L-glutamine, 1% Anti-Anti and 1mM sodium pyruvate
BT-549	RPMI supplemented with 10% FBS, 2 mM L-glutamine and 1% Anti-Anti
CAMA-1	RPMI supplemented with 10% FBS, 2 mM L-glutamine and 1% Anti-Anti
HCC-1806	RPMI supplemented with 10% FBS, 2 mM L-glutamine and 1% Anti-Anti
IMEC	DMEM/F-12 supplemented with 2 mM L-glutamine, 5 mg/ml Insulin, 10 ng/ml EGF, 0.5 µg/ml hydrocortisone and 1% Anti-Anti
JIMT-1	RPMI supplemented with 10% FBS, 2 mM L-glutamine and 1% Anti-Anti
MDA-MB-231	RPMI supplemented with 10% FBS, 2 mM L-glutamine and 1% Anti-Anti
MCF-7	RPMI supplemented with 10% FBS, 2 mM L-glutamine and 1% Anti-Anti
T47D	RPMI supplemented with 10% FBS, 2 mM L-glutamine and 1% Anti-Anti
ZR.75.1	RPMI supplemented with 10% FBS, 2 mM L-glutamine and 1% Anti-Anti

Table 2.5 Cell lines and culture media used in this thesis

Cell line	Subtype	Triple Negative*	Source	Tumour Type	References
MCF7	Luminal		PE	IDC	Kao et al., 2009 Neve et al., 2006
HCC-1806	NA	+	PT	SqC	Chavez et al., 2010
T47D	Luminal		PE	IDC	Kao et al., 2009 Neve et al., 2006
JIMT-1	Basal-like		PE	IDC	Koninki et al., 2010 Tanner et al., 2004
CAMA-1	Luminal		PE	AC	Neve et al., 2006
BT-549	Basal		PT	IDC	Chavez et al., 2010 Kao et al., 2009 Neve et al., 2006
MDA-MB-231	Basal	+	PE	AC	Chavez et al., 2010 Neve et al., 2006
ZR.75.1	Luminal	+	AF	IDC	Kao et al., 2009 Neve et al., 2006

AC, adenocarcinoma; AF, ascites fluid; IDC, invasive ductal carcinoma; NA, not available; PE, pleural effusion; PT, primary tumour; SqC, squamous carcinoma; +, positive for triple negative breast cancer

* Triple Negative status: HER2 overexpression and lacks expression of hormone receptors (estrogen and progesterone)

Table 2.6 Features of the breast cancer cell lines studied in this thesis

Subtype, source and clinical features of the breast cancer cell lines used in this thesis are indicated. Information on the cell lines was obtained from the indicated references.

2.2.6 Freezing/ thawing of cell lines

Cells were grown in a 75 cm^2 flask until 70% confluent and were trypsinised (as described above) and resuspended in RPMI media supplemented with 10% FBS to inactivate trypsin. The cell suspension was then centrifuged at 1700 rpm for 3 min and the resultant cell pellet was resuspended in 4 mls of 90% FBS/ 10% DMSO and transferred into a cyro vial (1 ml aliquots per 1.5 ml cyro vial). The cyro vials were stored in a Mr Frosty at -80 ˚C for 24-48 hr prior to long term storage in liquid nitrogen. Frozen cells were thawed at room temperature for 5 min and resuspended in 10 ml of relevant culture media (Table 2.5) and the cell suspension was then transferred into a 25 cm^2 flask.

2.2.7 Cell counting

Cell suspension was mixed 1:1 with trypan blue (0.4%) and cells were then counted using a Countess cell counter (Life technologies) according to the manufacturer's instructions.

2.2.8 Cell growth assays

The effect of short interfering RNA (siRNA)-mediated RNMT depletion on the proliferation of cells was established by seeding cells at equivalent density (2.5 X 10^5 cell/ well in a 6 well plate) and immediately transfecting cells with 3 independent siRNAs targeting RNMT, or a non-targeting siRNA. Cells were counted in triplicate on four consecutive days (1, 2, 3, 4 days post-

seeding/siRNA transfection) using a cell counter. Culture media was replenished 48 hr post-plating/siRNA transfection.

2.2.9 Cell doubling time

The cell doubling time for each cell line was established by plating cells at equivalent density (2.5 X 10^5 cell/ well in a 6 well plate) and cells were counted in triplicate on four consecutive days (1, 2, 3, 4 days post-plating) using a cell counter and the cell doubling time (hr) was calculated.

2.2.10 siRNA transfection

siRNA-mediated RNA-Interference (RNAi) was used to deplete RNMT expression levels throughout this thesis. All cells were transfected with siRNA using Lipofectamine RNAiMax transfection reagent according to the manufacturer's instructions. For a 6 cm dish, 0.45 nmol of siRNA was transfected with 9 µl of Lipofectamine RNAiMax and 480 µl serum-free DMEM media. For a well in a 6 well dish, 0.16 nmol of siRNA was transfected with 3 µl of Lipofectamine RNAiMax and 200 µl serum-free DMEM media. All siRNAs used in this thesis are shown in Table 2.7.

All cell lines used were seeded at 2.5 X 10^5 cells per well/ 6 well dish, or 6 X 10^5 per 6 cm dish, and were immediately transfected with siRNA. For experiments requiring longer than 48 hr siRNA transfection, media was replenished at 48 hr with the relevant culture media (Table 2.5).

siRNA target	Catalogue details	Sequence
Non-targeting	D-001210-03-50	-
RNMT 1	D-019525-01-0050	GUUCUAAACUUGUCUCUGA
RNMT 2	D-019525-02-0050	GCAAAUAUGACUUCAACUU
RNMT 3	D-019525-03-0050	GAAUUAACAAGCUAGUUG

Table 2.7 siRNAs used in this thesis

2.2.11 DNA transfection using lipofectamine 2000

Phoenix cells were transiently transfected with DNA vectors using lipofectamine 2000 according to the manufacturer's instructions. For a 10 cm dish, 5 µg of DNA was transfected with 15 µl of lipofectamine 2000 and 500 µl of serum-free DMEM media. Cells were seeded at 5 X 10^5/ 10 cm dish and left to adhere for 24 hr prior to DNA transfection. Media was replenished with culture media 6 hr post-DNA transfection.

2.2.12 Treatment of cells with inhibitors

The inhibitors used in this thesis (GDC-0941 and Staurosporine) were produced by the DSTT and were dissolved in DMSO. For different concentrations of inhibitors, the 10 mM stock was diluted further with DMSO and added to the cell culture media. For the control treatment, an equal volume of DMSO was added to the culture media.

To assess the combined effect of siRNA-mediated RNMT depletion and pharmacological PI3K inhibition on the proliferation of T47D cells, cells were seeded at 2.5 X 10^5 cells/ well (in a 6 well dish) and transfected with siRNA

targeting RNMT, or non-targeting siRNA. 4 hr post-siRNA transfection, cells were treated with GDC-0941 (0.05 μM), or an equal volume of DMSO. 72 hr post-siRNA transfection, cells were counted using a cell counter, or protein extracted for Immunoblot analysis.

As a positive control for the cleaved-PARP Immunoblot experiments, cells were treated with 0.1 μM straurosporine for three hours prior to cell lysis. Cells were then lysed and cell lysates subject to immunoblot analysis.

2.2.13 Generation of stable cell lines

HCC-1806, ZR.75.1 and IMEC cells were stably transfected using the retroviral transduction method. Firstly, Phoenix cells were transfected with DNA vectors using lipofectamine 2000 according to the manufacturer's instructions to generate infectious retroviruses. 48 hr post-transfection, 10 ml of virus-containing media was passed through a 45 micron filter and supplemented with 5 μg/ ml of polybrene and this was then used to infect a 10 cm dish of cells. Successfully transduced cells were selected for using 0.5 mg/ml of Geneticin.

2.2.14 Rescue of siRNA-mediated RNMT depletion

A siRNA-resistant full-length RNMT construct was made by introducing several silent mutations which alter the nucleotide sequence, but do not alter the amino acid sequence, into a GFP-tagged full-length RNMT construct (Table 2.4). Then HCC-1806 cells were retrovirally transduced to stably express either wild-type, or siRNA-resistant, GFP-tagged full-length RNMT.

2.2.15 Cell lysis

Cell lysis was performed at 4 ˚C to avoid protein degradation. Culture media was removed and cells were washed once with ice-cold PBS. Cells were lysed with ice-cold F buffer. For Immunoblot analysis of phosphorylated proteins, the lysis buffer was further supplemented with Phosphatase inhibitors (cocktail mixture 2 + 3) to avoid dephosphorylation of proteins. Cell lysates were collected by scraping and the soluble fraction collected following 13,000 rpm for 10 min at 4˚C. Protein concentration was determined using the Bradford method.

2.2.16 Protein concentration determination

Protein concentration was determined using the Bradford method (Bradford, 1976). To generate a standard curve, dilutions of BSA standard (0.5, 1, 1.5 μg of BSA) were made in F-buffer and 2 μl of the BSA dilutions, or F-buffer only (blank), were added to 200 μl of 1X Bradford Protein assay reagent in a 96-well plate. Absorbance was measured at 595 nm using a plate reader. A standard curve was obtained by plotting the absorbance reading against the concentration of BSA and a line equation determined. To determine the protein concentration of samples, samples were diluted in water and analysed the same as described above. The linear equation of the standard curve was used to establish the protein concentration of the sample. All Bradford measurements were performed in duplicate.

2.2.17 Immunoprecipitation (IP)

All IP steps were performed at 4˚C to avoid protein degradation. For all Myc-tagged exogenously expressed proteins, 1.5 mg of protein lysates were pre-cleared with agarose beads for 30 min on a rotating wheel. The agarose beads were then pelleted by centrifugation at 3000 rpm for 1 min. The pre-cleared supernatant was transferred to a new eppendorf tube and incubated with 1.5 µg of anti-E910 (Myc tag) antibody for 2 hr on a rotating wheel. Agarose beads were then added to the mixture for an additional 30 min. The IPs were pelted by centrifugation at 3000 rpm for 1 min, the supernatant discarded and the beads washed 3 times with 1 ml of F buffer. Immunoprecipitated proteins were eluted in 50 µl of 1X Loading dye and 15 µl were analysed by SDS-PAGE. Loading dye was also added to the IP inputs, which were analysed along with the IPs by SDS-PAGE.

2.2.18 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Each resolving gel contained 400 mM Tris (pH 8.8), 0.1% SDS, 0.1% APS and 8-15% acrylamide. The amount of protein lysate analysed and the percentage of acrylamide used in the resolving gels for the immunoblot analysis of specific proteins is shown in Table 2.8.

Each stacking gel contained 400 mM Tris (pH 6.8), 0.1% SDS, 0.1% APS and 5% acrylamide. 0.01% TEMED was added to initiate the polymerisation reaction. Homemade gels were resolved in a Mini-PROTEAN Tetra cell (Bio-Rad) using SDS Running buffer.

4-12% NuPAGE Novex gradient gels were used for some experiments and the use of these gels are indicated in the figure legends. NuPAGE Novex gels were resolved in a X-Cell SureLock Mini-Cell (Life Technologies). MOPS running buffer (Novex) was used for resolving proteins larger than 30 kDa, whereas MES running buffer (Novex) was used for smaller proteins.

Protein analysed	Protein analysed (µg)	% of acrylamide gel
Akt	15	8 or 4-12
Actin	5	8 or 4-12
cleaved-PARP	20	6
c-Myc	15	8 or 4-12
eIF4E-BP1	20	4-12
P70 S6 kinase	20	4-12
RAM	15	15 or 4-12
RNMT	5	8 or 4-12
Tubulin	5	8

Table 2.8 Proteins analysed by immunoblotting in this thesis.

Protein samples were denatured in 1X Laemmli sample buffer at 95˚C for 5 min and centrifuged 13000 rpm for 2 min at room temperature. Protein samples (5-20 µg of total protein) (Table 2.8), or 15 µl of immunoprecipitate, were loaded per lane and 4 µl of protein standards, which have apparent molecular weights of 180, 115, 82, 64, 49, 37, 26, 19, 15 kDa, were loaded in the first lane of each gel. Gels were resolved at 160 V until the samples reached the bottom of the gel.

2.2.19 Transfer of proteins to PVDF membrane

Proteins separated by SDS-PAGE were transferred onto PVDF membrane by electro-transfer. The gel was placed on PVDF (which had been pre-activated by incubation with methanol for 5 min), which was then packed between 3 mm Wattman filter papers and sponges all submerged in transfer buffer. The sandwich was loaded into a mini-Trans-blot cell with the gel facing the cathode, and the PVDF membrane facing the anode, to ensure the transfer of the negatively charged proteins onto the PVDF membrane. The electro-transfer was carried out at 68 V for 1.5 hr.

2.2.20 Immunoblotting

Membranes were blocked with 5% milk, or 3% BSA, /TBS-Tween for 1 hr at room temperature to prevent non-specific binding of primary antibody. Membranes were incubated with primary antibody diluted in either 5 % milk, or 3% BSA, /TBS-Tween overnight at 4˚C. All commercial antibodies were diluted in 3 % BSA/ TBS-Tween and the antibody dilution conditions used for in-house antibodies are shown in Table 2.3. Membranes were then incubated with HRP-conjugated secondary antibody diluted in 5 % milk/ TBS-Tween for 1 hr at room temperature and membranes were subsequently washed 3 times in TBS-Tween. Finally, the membranes were incubated with Super Signal West Pico Chemiluminescent substrate for 2 min and signal exposed to x-ray film for varying lengths of time and developed in an automatic processor.

2.2.21 [35S]-amino acid labelling

Cells were incubated for 20 min at 37˚C with pre-warmed media containing 11 µCi/ ml protein labelling mix (containing 35S-methionine and 35S-cysteine). Cells were washed once with ice-cold PBS and lysed in F-buffer. Protein lysates were then resolved by SDS-PAGE in a 10% polyacramide gel. A polyacramide gel loaded with 6 µg of protein was stained with Simply Blue stain and used to assess equal loading of protein in each lane. In order to quantify the incorporation of [35S]-labelled amino acids into nascent proteins, a polyacramide gel loaded with 12 µg of protein was desiccated and then underwent autoradiography.

2.2.22 Coomassie staining of polyacrylamide gels

Following SDS-PAGE, gels were washed 3 times in distilled water and stained with SimplyBlue stain for 30 min. After staining, gels were washed in distilled water until the gel background was distained.

2.2.23 Desiccation of polyacrylamide gels

For [35S]-amino acid labelling experiments, gels were placed on 2 sheets of pre-wetted Whatman 3mm filter paper and covered with cellophane wrap. The gels were dried in a Gel Air drier for 1.5 hr and then subject to autoradiography.

2.2.24 Autoradiography

The radioactive signal was recorded on to a phosphor screen and an image of the radioactive signal was created using a phosphorimager. The AIDA imager analyser software was used to obtain quantitative measurement of the radioactive signal.

2.2.25 Phenol/chloroform extraction of RNA

Phenol/ chloroform extraction was used to remove contaminating proteins and DNA from RNA solutions. Phase partitioning of nucleic acids is dependent on the pH of the phenol: at pH ≤ 7, the DNA moves into the organic phase and interphase, whereas the RNA remains in the aqueous phase. Phenol (pH 6.6) was used for phenol/chloroform extraction in this thesis to ensure the selective purification of RNA. An equal volume of phenol/ chloroform was added to the DNA/ RNA/ protein solution and vortexed for 10 sec to mix the phases. The aqueous and organic phases were separated by centrifugation at 13000 rpm for 10 min at 4˚C. The aqueous phase containing the RNA solution was then transferred to a new tube.

2.2.26 RNA precipitation

Following phenol/chloroform extraction, the aqueous phase was combined with 2 volumes of 100% ethanol containing 0.5 μM ammonium acetate, and then incubated overnight at 20˚C. The precipitate was then pelted by centrifugation at 13000 rpm for 1 hr. The RNA pellet was washed twice with 70% ETOH and

left to air dry for 1 hr. Finally, the RNA pellet was resuspended in 50 µl of DEPC-treated H_2O.

2.2.27 *In vitro* cap methyltransferase assay

pGEM CEM was linearised using EcoRI and then purified using a Qiagen PCR purification column according to the manufacturer's instructions. T7 RNA polymerase (Promega) was used according to the manufacturer's instruction to *in vitro* transcribe RNA from pGEM CEM. Briefly, 500 ng of linerased pGEM CEM was incubated with T7 RNA polymerase, 250 mM NTPs and Promega transcription buffer. The *in vitro* transcribed RNA was then purified using phenol chloroform and the ethanol-precipitated pellet resuspended in 50 µl of DEPC-treated H_2O. The *in vitro* transcription steps described above were performed by Thomas Gonatopoulous-Pournatzis, a previous PhD student in the Cowling laboratory.

To add a [α-32P]- labelled guanosine cap to the 5' end of RNA, 200 ng of RNA *in vitro* transcribed from pGEM CEM was incubated with 20 µCi of [α-32P]-guanosine 5'-triphosphate (GTP), 2 µg recombinant RNGTT (human capping enzyme), 10% RNGTT buffer and 40 U RNasin at 37˚C for 1 hr. The *in vitro* transcribed capped RNA was purified using phenol chloroform and the ethanol-precipitated pellet resuspended in 50 µl of DEPC-treated H_2O.

For the *in vitro* cap methylation reaction, protein lysates (0.25 µg, 0.5 µg or 1 µg) prepared by F buffer protein extraction were incubated with 2 mM SAM, 20 U RNasin, 1% MT buffer and 1/100th of purified *in vitro* transcribed capped RNA at 37˚C for 10 min. The resultant RNA was purified using Phenol Chloroform and the ethanol-precipitated pellet was resuspended in 4 µl of 50 mM Na

Acetate (pH 5.5). The RNA was subsequently incubated with 20% P1 nuclease at 37˚C for 1 hr to cleave guanosine cap (GpppG), or methyl guanosine cap (m7GpppG), from the transcript. [α-32P]-labelled GpppG, or [α-32P]-labelled m7GpppG, were resolved by Thin layer chromatography (TLC) (using PEI cellulose plates in 0.4 M ammonium sulphate). The TLC was visualised using autoradiography and quantified using AIDA imager analyser.

The concentration of methyl capped (m7GpppG) RNA was calculated based on an input of 200ng of RNA into the [α-32P]-GTP capping reaction and assuming a 100% [α-32P]-GTP capping efficiency and a 100% recovery of RNA at the subsequent phenol chloroform and ethanol precipitation steps.

2.2.28 Statistical analysis

Statistical significance was assessed by one way analysis of variance (ANOVA) followed by Dunnett's, or Tukey's, multiple comparison tests using GraphPad Prism 5.0. P-value ≤ 0.05 was considered to a significant difference; P-value ≤ 0.05 is denoted with *; P-Value ≤ 0.01 is denoted with **; P-Value ≤ 0.001 is denoted with ***.

Chapter 3: A subset of breast cancer cell lines exhibit enhanced dependency on RNMT for survival

3.1 Introduction

Eukaryotic RNA polymerase II (RNA Pol II) transcripts are uniquely modified at the 5' terminus with the addition of a 7-methylguanosine cap (methyl cap) structure. From yeast to humans, the methyl cap is required for efficient gene expression and cell viability. The methyl cap is necessary for the translation of most mRNAs and it has also been shown to promote transcription, splicing, polyadenylation, and nuclear export of mRNAs (Cowling, 2009b, Furuichi and Shatkin, 2000).

Formation of the methyl cap occurs predominantly, if not entirely, co-transcriptionally. Synthesis of the methyl cap is dependent on the sequential enzymatic activities of an RNA triphosphatase, RNA guanylyltransferase and RNA methyltransferase. The RNA triphosphatase removes the terminal phosphate of the transcript and the RNA guanylyltransferase catalyses the addition of guanosine monophosphate (GMP) to form the cap structure. To finalise the methyl cap structure, the RNA methyltransferase methylates the guanosine cap at the N-7 position (Cowling, 2009b, Furuichi and Shatkin, 2000).

The human cap methyltransferase, RNMT, catalyses the methylation of the guanosine cap (Pillutla et al., 1998b) and our laboratory has recently discovered that RAM (RNMT activating mini-protein), a novel interacting

protein of RNMT, is essential for RNMT expression and activity (Gonatopoulos-Pournatzis et al., 2011). Several recent studies have demonstrated that mRNA cap methylation is a regulated process (Fernandez-Sanchez et al., 2009a, Cole and Cowling, 2009, Jiao et al., 2010).

Deregulated mRNA translation is associated with cancer. Despite being essential for cell viability, emerging data suggest that targeting mRNA translation initiation is an effective anti-cancer strategy (Blagden and Willis, 2011, Silvera et al., 2010). In this thesis, I set out to investigate the suitability of RNMT as a therapeutic target in breast cancer. Expression of the human mRNA cap methyltransferase, RNMT, is rate-limiting for efficient mRNA translation and cell proliferation (Cowling, 2009a, Chu and Shatkin, 2008). Intriguingly, several lines of evidence from our laboratory suggest there is strong biological rationale to explore RNMT as a therapeutic target in breast cancer. Firstly, the oncogene c-Myc can upregulate cap methylation in mammary epithelial cells and this process is required for c-Myc-driven protein synthesis and cell proliferation. Secondly, inhibition of cap methylation is synthetic lethal with elevated c-Myc expression (Fernandez-Sanchez et al., 2009b). Thirdly, experimental overexpression of RNMT is sufficient to transform mammary epithelial cells (Cowling, 2009a). However, it remains unknown whether cancer cells exhibit an enhanced dependency on RNMT for proliferation, in comparison to non-cancerous cells. To assess RNMT as a therapeutic target in breast cancer, the initial aim of this project was to investigate the effect of RNMT depletion on the proliferation of breast cancer cells and non-transformed mammary epithelial cells. It is important to note that the inhibition of an effective therapeutic target should reduce the proliferation of cancerous cells, but cause limited toxicity to non-cancerous cells.

3.2 Results

3.2.1 siRNA-mediated RNMT depletion does not significantly impair the proliferation of non-transformed mammary epithelial cells

I first sought to establish whether RNMT depletion affected the proliferation of immortalised mammary epithelial cells (IMECs). The IMEC cell line was originally established by overexpressing the catalytic subunit of human telomerase in primary human mammary epithelial cells. In addition, IMECs have previously been shown to be immortalised, but non-transformed (DiRenzo et al., 2002). When this project was initiated there were no specific inhibitors against RNMT activity, therefore, I explored whether RNMT expression could be depleted in IMECs using siRNA-mediated RNA-Interference. Cells were transfected with three independent siRNAs targeting RNMT, or a non-targeting siRNA. 72 hr post-siRNA transfection, cells were lysed and protein lysates were immunoblotted with antibodies against RNMT and RAM to assess their expression. RNMT and RAM antibodies have previously been shown to be specific against endogenous RNMT and RAM protein expression, respectively (Gonatopoulos-Pournatzis et al., 2011). As shown in Figure 3.1A, the three independent siRNAs reduced endogenous RNMT protein expression. It has previously been reported that expression of RAM protein is dependent on RNMT protein expression, and vice and versa (Gonatopoulos-Pournatzis et al., 2011). Consistent with this, an efficient reduction in RNMT protein also caused a depletion of RAM protein using the three independent siRNAs (Figure 3.1A). It should be noted that detectable levels of both endogenous RNMT, and RAM, were observed on longer immunoblot exposures.

In order to investigate the effect of RNMT depletion on the ability of IMECs to proliferate, cells were transfected with three independent siRNAs targeting RNMT, or a non-targeting siRNA, and then counted on four consecutive days. Strikingly, as shown in Figure 3.1B, RNMT depletion did not significantly affect the proliferative capacity of IMECs. Reassuringly, each of the three independent siRNAs targeting RNMT produced the same result.

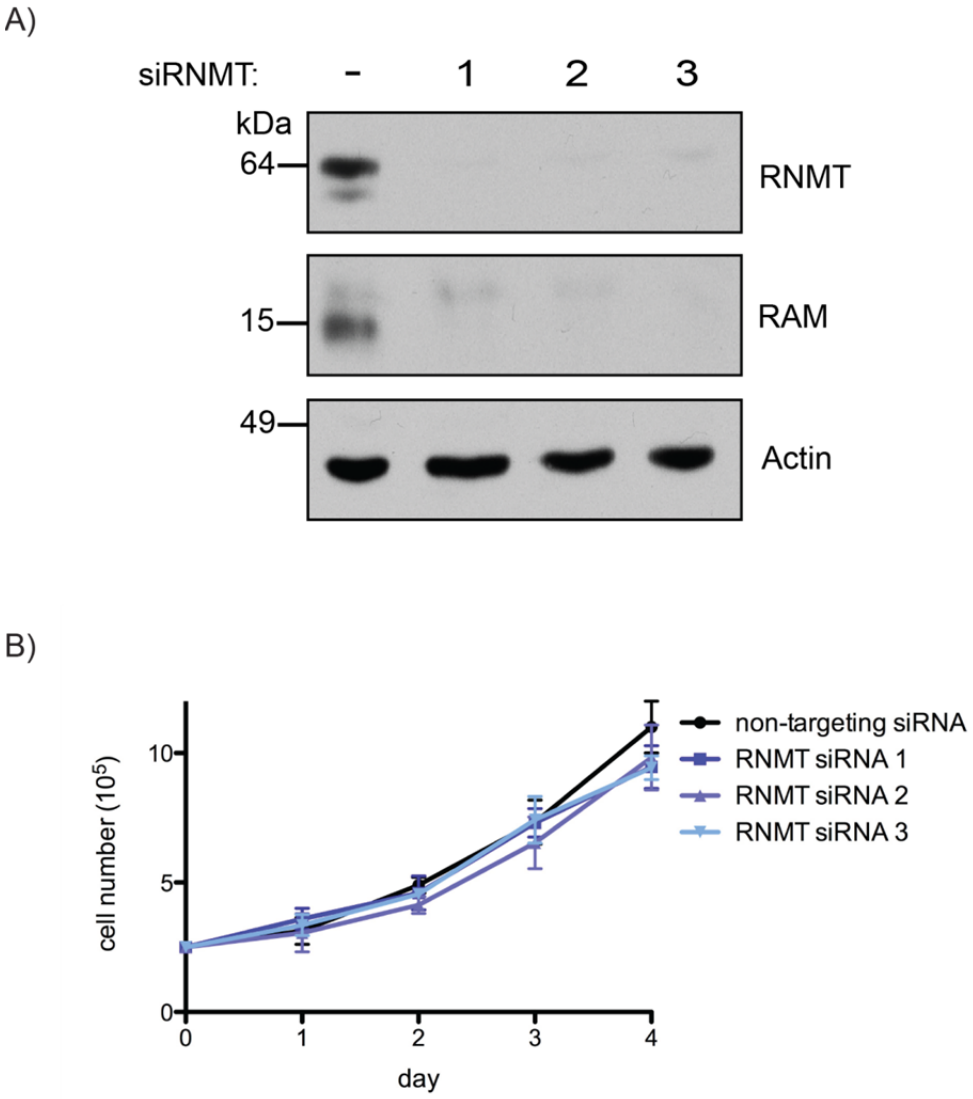


Figure 3.1 The proliferative capacity of IMECs is not significantly affected by siRNA-mediated RNMT depletion

(**A**) Cells were transfected with 3 independent siRNAs targeting RNMT (1, 2, 3), or a non-targeting siRNA (-), for 72 hr. Protein lysates were analysed by immunoblotting with the indicated antibodies. Actin serves as loading control. (**B**) Equal number of cells were plated and transfected with siRNAs targeting RNMT (1, 2, 3) (day 0), or a non-targeting siRNA, and cells were counted on 4 consecutive days (day 1, 2, 3, 4) using a cell counter. Error bars indicate SD. Result is representative of two independent biological replicates.

3.2.2 RNMT depletion impairs the proliferation of a subset of breast cancer cells

To further assess the suitability of RNMT as a therapeutic target in breast cancer, I next investigated whether siRNA-mediated RNMT depletion impaired the proliferation of a panel of eight breast cancer cells lines (MCF7, HCC-1806, JIMT-1, T47D, CAMA-1, BT-549, MDA-MB-231, ZR.75.1). The key features (subtype, source, clinical features) of the breast cancer cell panel are shown in Table 7.1 in the appendix. Cells were transfected with three independent siRNAs targeting RNMT, or a non-targeting siRNA, and lysed 72 hr post-siRNA transfection. Protein lysates were then immunoblotted with antibodies against RNMT, and RAM, to assess RNMT depletion. As shown in Figure 3.2, transfection of three RNMT siRNAs, resulted in a reduction of RNMT expression in all the breast cancer cells tested. As observed in IMECs (Figure 3.1A), transfection of RNMT siRNA resulted in a reduction in RAM expression. The immunoblots in Figure 3.2, show that both RNMT and RAM are depleted to similar levels across the different breast cancer cell lines. It should be noted that on longer immunoblot exposures, endogenous RNMT and RAM was detectable for all the breast cancer cell lines tested.

To investigate the impact of RNMT depletion on the proliferation of the breast cancer panel, cells were transfected with three independent siRNAs targeting RNMT, or a non-targeting siRNA, and were then counted on four consecutive days (Figure 3.3). Interestingly, RNMT depletion was found to impair the proliferation of four breast cancer cell lines (MCF7, HCC-1806, JIMT-1 and T47D), whereas not significantly affecting the others (CAMA-1, BT-549, MDA-MB-231 and ZR.75.1). For the following cell lines: T47D, CAMA-1, BT-549, MDA-MB-231 and ZR.75.1, transfection of the three independent RNMT

siRNAs produced a similar effect on the proliferation of each cell line (Figure 3.3.). However, RNMT siRNA 2 was not as efficient at reducing cellular proliferation as RNMT siRNA 1 and 3 (Figure 3.3) for the following cell lines: MCF7, HCC-1806 and JIMT-1.

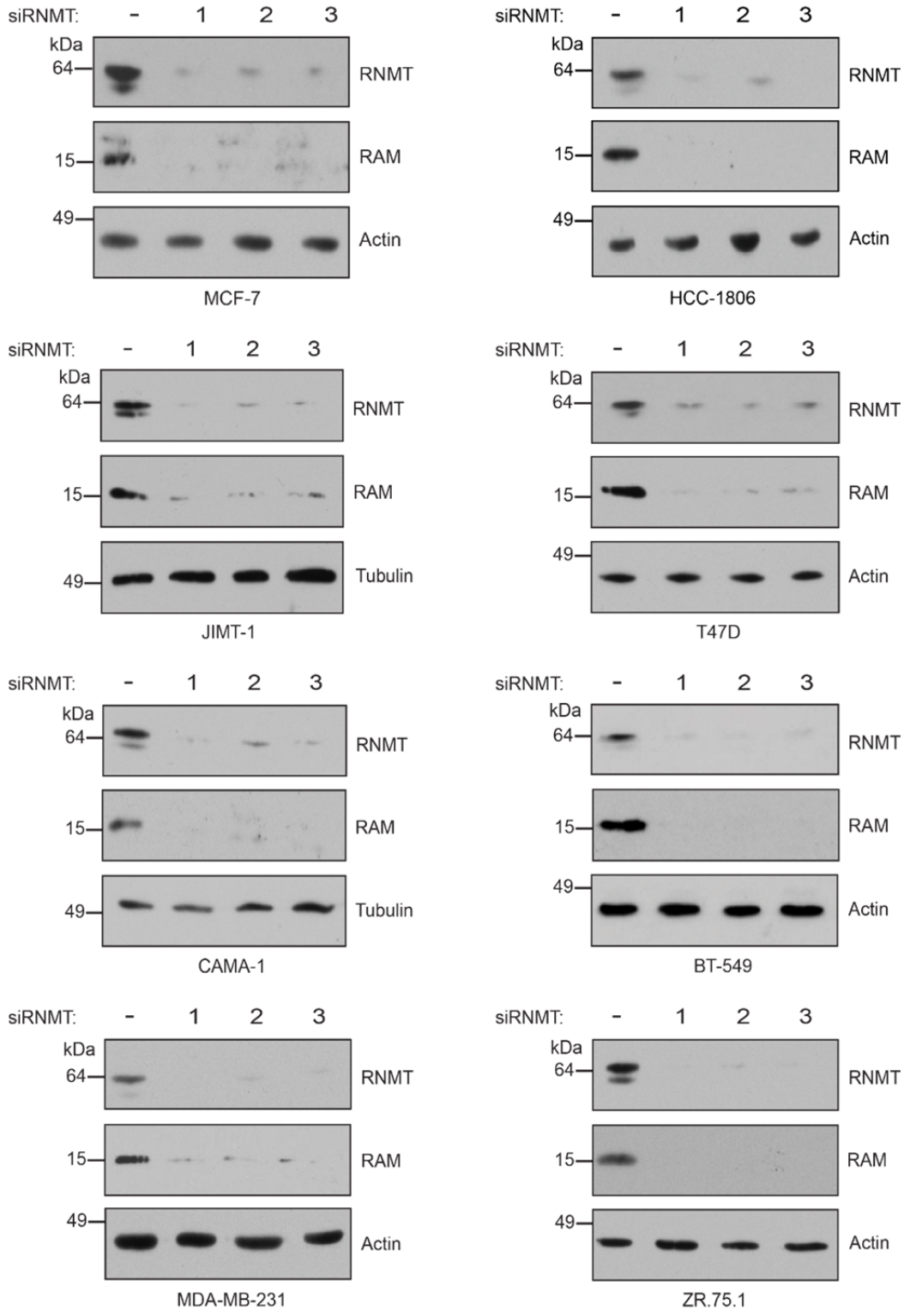
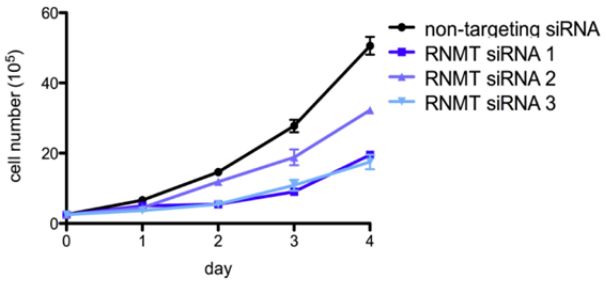


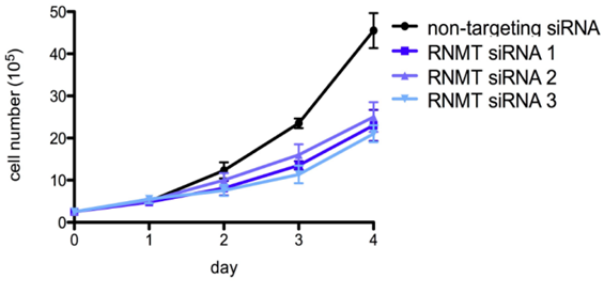
Figure 3.2 RNMT protein levels are effectively and equivalently depleted using siRNA-mediated RNA Interference in breast cancer cells

Cells were transfected with 3 independent siRNAs targeting RNMT (1, 2, 3), or a non-targeting siRNA (-), for 72 hr. Protein lysates were analysed by immunoblotting with the indicated antibodies. Tubulin, or Actin, serve as loading controls. The cell lines tested are indicated below the loading controls. Result is representative of two independent biological replicates.

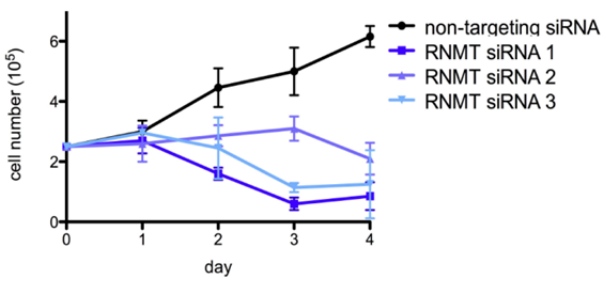
HCC-1806



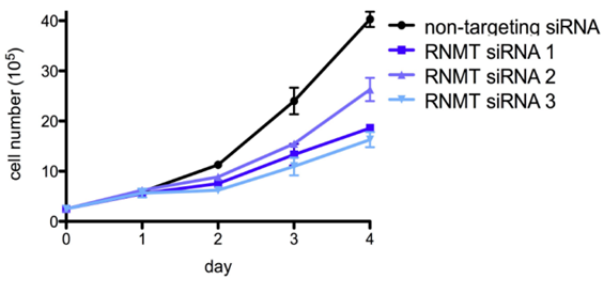
T47D



MCF-7



JIMT-1



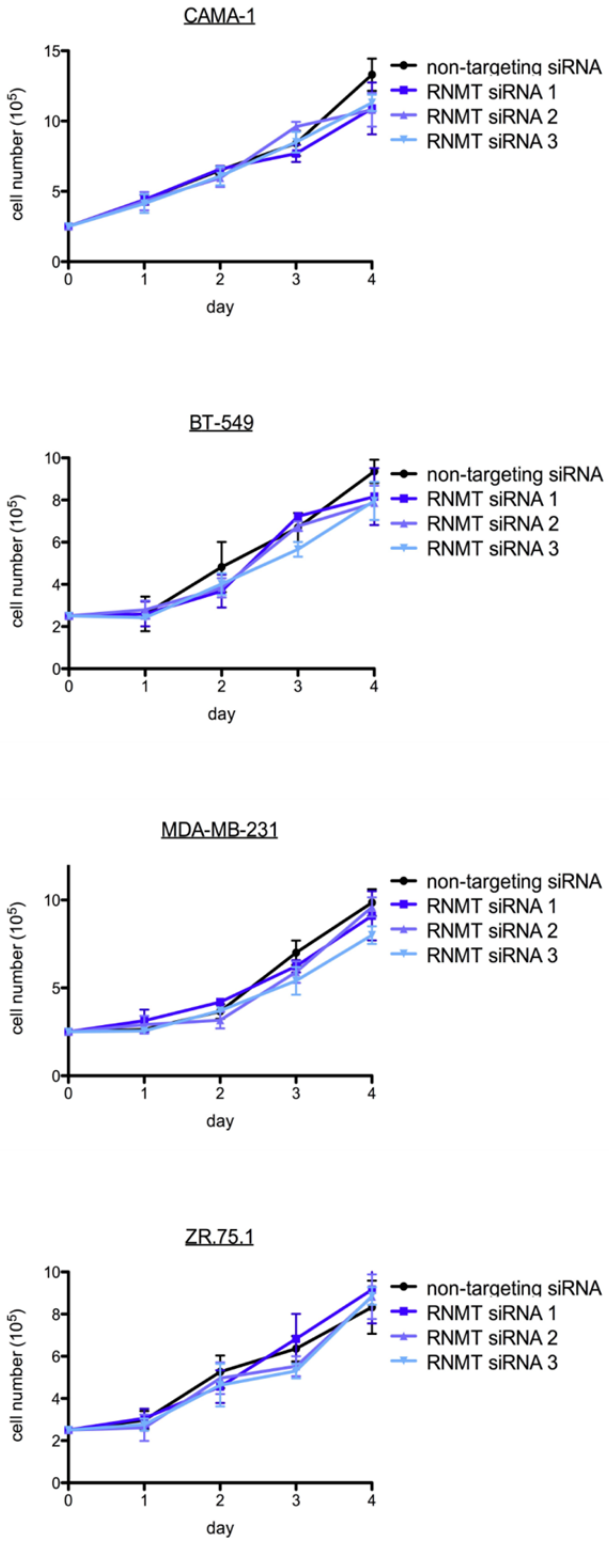


Figure 3.3 RNMT depletion impairs the proliferation of a subset of breast cancer cell lines

Equal number of cells were plated and transfected with siRNAs targeting RNMT (1, 2, 3) (day 0), or a non-targeting control siRNA, and cells were counted in triplicate on 4 consecutive days (day 1, 2, 3, 4) using a cell counter. Graph shows the mean cell number against days of growth and SD. Result is representative of two independent biological experiments.

3.2.3 A subset of the breast cancer cell lines tested exhibit enhanced dependency on RNMT for survival

If the depletion of RNMT can selectively inhibit the proliferation of breast cancer cells, it would provide the first evidence to suggest that RNMT would make a good therapeutic target in breast cancer. The afore-mentioned experiments (Figure 3.1 and Figure 3.3) showed that equivalent RNMT depletion impaired the proliferation of four breast cancer cell lines (MCF7, HCC-1806, JIMT-1 and T47D), whereas it did not significantly affect the proliferation of the other four breast cancer cell lines tested (CAMA-1, BT-549, MDA-MB-231 and ZR.75.1) and non-transformed mammary epithelial cells (IMECs). In an attempt to directly compare the ability of RNMT depletion to inhibit the proliferation of the breast cancer panel (MCF7, HCC-1806, JIMT-1, T47D, CAMA-1, BT-549, MDA-MB-231, ZR.75.1) and IMECs, cells were transfected with siRNA targeting RNMT, or a non-targeting siRNA, and cells were counted 96 hr post-siRNA transfection (Figure 3.4). It should be noted that previous experiments had shown that RNMT could be efficiently depleted in all the cell lines tested (Figure 3.1A and Figure 3.2). MCF7, HCC-1806, JIMT-1 and T47D exhibit a significant decrease in cell number in response to RNMT depletion, relative to IMECs ($P \leq 0.001$, ANOVA followed by Dunnett's multiple comparison test). Thus, I concluded these four cell lines were "sensitive to RNMT depletion" (Figure 3.4). In contrast, CAMA-1, BT-549, MDA-MB-231 and ZR.75.1 exhibit no significant decrease in cell number in response to RNMT depletion ($P > 0.05$, ANOVA followed by Dunnett's multiple comparison test). Thus, I concluded these four cell lines were "insensitive to RNMT depletion (Figure 3.4). These results show that targeting RNMT can

selectively inhibit the proliferation of a subset of breast cancer cells, in comparison to non-transformed mammary epithelial cells.

During the comparison of sensitivity to RNMT depletion, a key issue was whether the differential dependency of breast cancer cells on RNMT for proliferation (Figure 3.3 and Figure 3.4) was simply due to differences in RNMT depletion levels between the cells “sensitive to RNMT depletion” and those cells “insensitive to RNMT depletion”. To directly compare RNMT depletion levels between the two groups of cells, two representative cell lines from each group were transfected (MCF7, HCC-1806, BT-549 and ZR.75.1) with siRNA targeting RNMT, or a non-targeting siRNA, for 48 hr. Immunoblot analysis was then performed on protein lysates using antibodies that detect RNMT and RAM. This revealed that depletion of RNMT protein was relatively equivalent across the four cell lines tested. Furthermore, consistent with an equivalent reduction in RNMT levels, the depletion of RAM protein was also relatively equivalent across the four cell lines tested (Figure 3.5). These observations indicate that RNMT depletion is comparable in the breast cancer cells “sensitive to RNMT depletion” and in those “insensitive to RNMT depletion”.

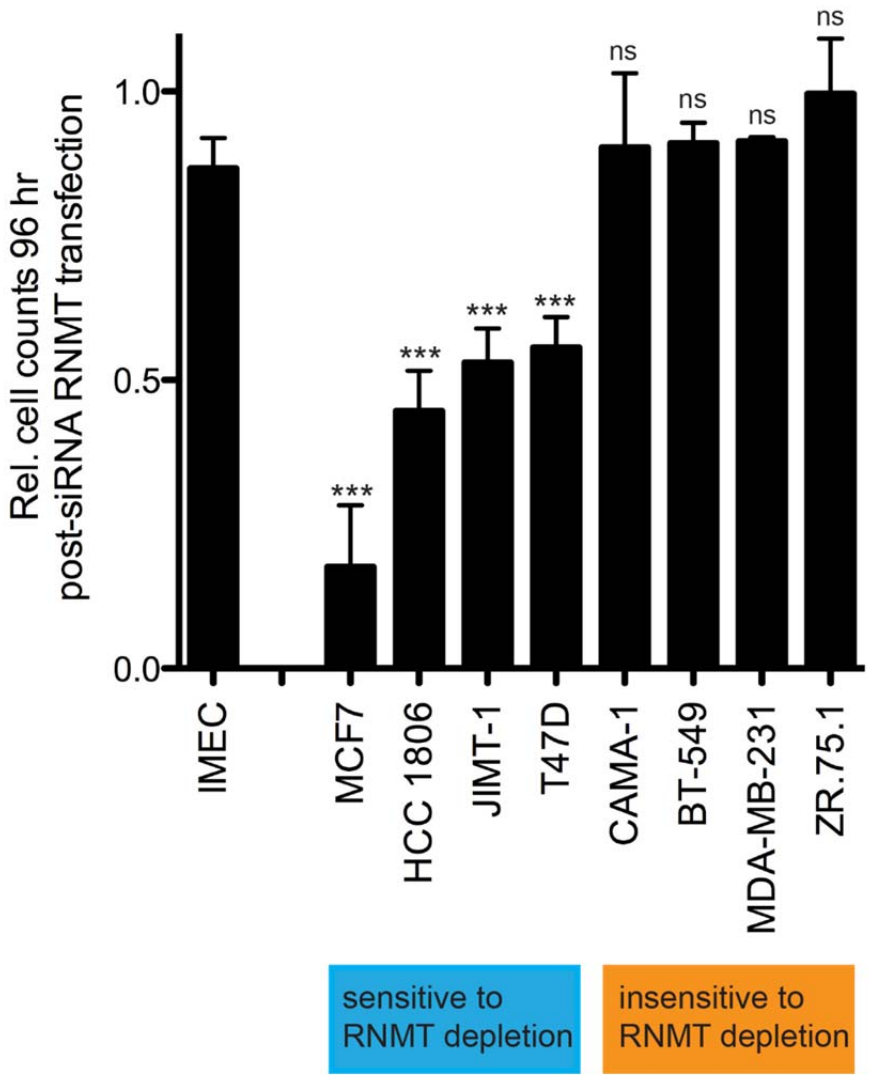


Figure 3.4 A subset of breast cancer cell lines are significantly more dependent on RNMT for proliferation in comparison to IMECs

Cells were transfected with siRNA RNMT 1, or non-targeting control siRNA, for 96 hr and counted using a cell counter. Bar chart depicts mean number of RNMT siRNA transfected cells relative to non-targeting siRNA transfected cells. SD for 3 independent biological replicates are indicated. Statistical significance was assessed by ANOVA followed by Dunnett's multiple comparison test using GraphPad Prism 5.0. ns $P > 0.05$, no significance difference compared to IMEC; *** $P \leq 0.001$.

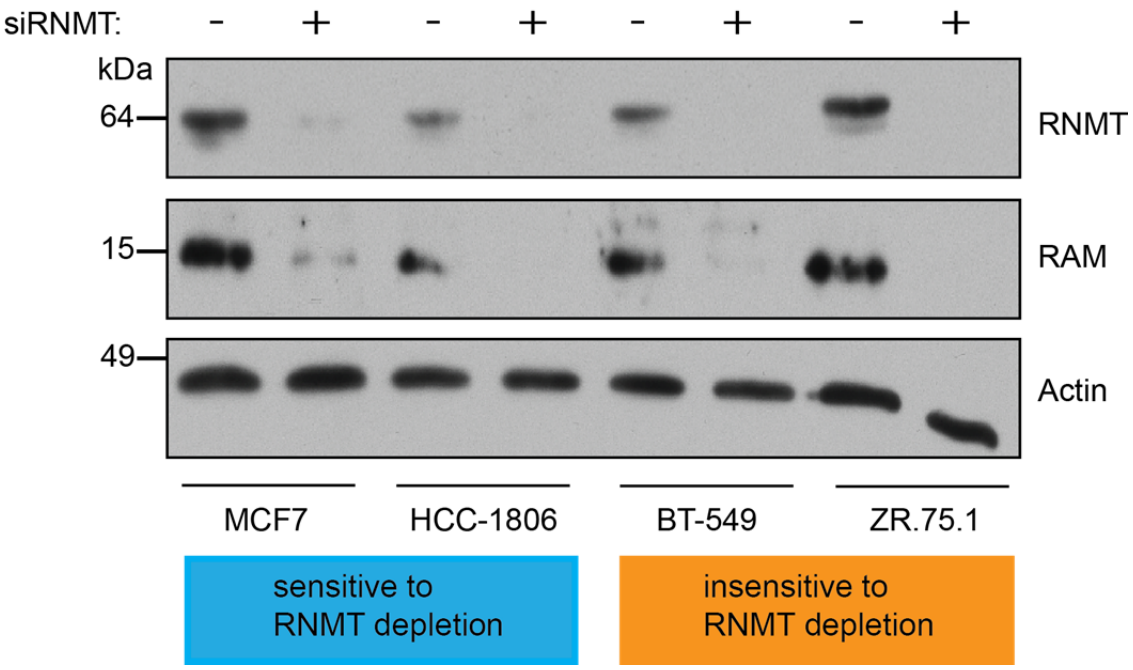


Figure 3.5 siRNA-mediated depletion of RNMT is relatively equivalent between the breast cancer cell lines tested

Cells were transfected with siRNA targeting RNMT (+) or a non-targeting siRNA (-), for 48 hr. Resulting cell lysates were analysed by immunoblotting with the indicated antibodies. Actin blot serves as loading control. Result is representative of 2 independent biological replicates. The sensitivity of breast cancer cells to RNMT depletion is indicated.

3.2.4 Rescue of the proliferative defect induced by RNMT depletion

To establish whether the impaired proliferation induced by RNMT depletion (Figure 3.3 and Figure 3.4) was specifically due to a reduction in RNMT expression, or was simply a consequence of siRNA off target effects, a "rescue" experiment was carried out. A siRNA-resistant RNMT-GFP construct was made by introducing several silent point mutations which altered the nucleotide sequence, but did not alter the amino acid sequence, into a GFP-tagged full-length RNMT construct. For use in the rescue experiment, HCC-1806 cells stably expressing siRNA-resistant RNMT-GFP, or vector control, were made by retroviral transduction. To determine RNMT and RAM expression levels in response to endogenous RNMT depletion, cells were transfected with siRNA targeting RNMT, or a non-targeting siRNA, for 72 hr and an immunoblot analysis was carried out using antibodies against RNMT and RAM (Figure 3.6A). This revealed that endogenous RNMT was depleted equivalently in cells expressing RNMT-GFP, or vector control, and there was a corresponding reduction in endogenous RAM protein in cells expressing vector control. I was able to demonstrate that expression of exogenous RNMT-GFP maintained RAM expression in spite of endogenous RNMT depletion (Figure 3.6A). To test whether exogenous expression of RNMT-GFP could rescue the proliferative defect induced by endogenous RNMT depletion, cells were transfected with siRNA targeting RNMT, or non-targeting siRNA, and counted 72 hr post-siRNA transfection (Figure 3.6B). Consistent with previous experiments (Figure 3.3 and Figure 3.4), depletion of endogenous RNMT significantly inhibited the cell number by approximately 40%, in comparison to cells transfected with non-targeting siRNA ($P \leq 0.001$, ANOVA followed by Tukey's multiple comparison test). Importantly, cells expressing RNMT-GFP

had no significant decrease in cell number in response to endogenous RNMT depletion, in comparison to cells transfected with non-targeting siRNA ($P > 0.05$ ANOVA followed by Tukey's multiple comparison test). Thus, expression of exogenous RNMT-GFP rescued the reduction in cell number of RNMT depleted cells. It should be noted that, in comparison to cells expressing vector control, exogenous expression of RNMT-GFP did not alter the proliferation rate of HCC-1806 cells ($P > 0.05$ ANOVA followed by Tukey's multiple comparison test) (Figure 3.6B). Overall these results show that the impaired proliferation of RNMT depleted cells is indeed due to a reduction in RNMT expression, and is not simply a consequence of siRNA off target effects.

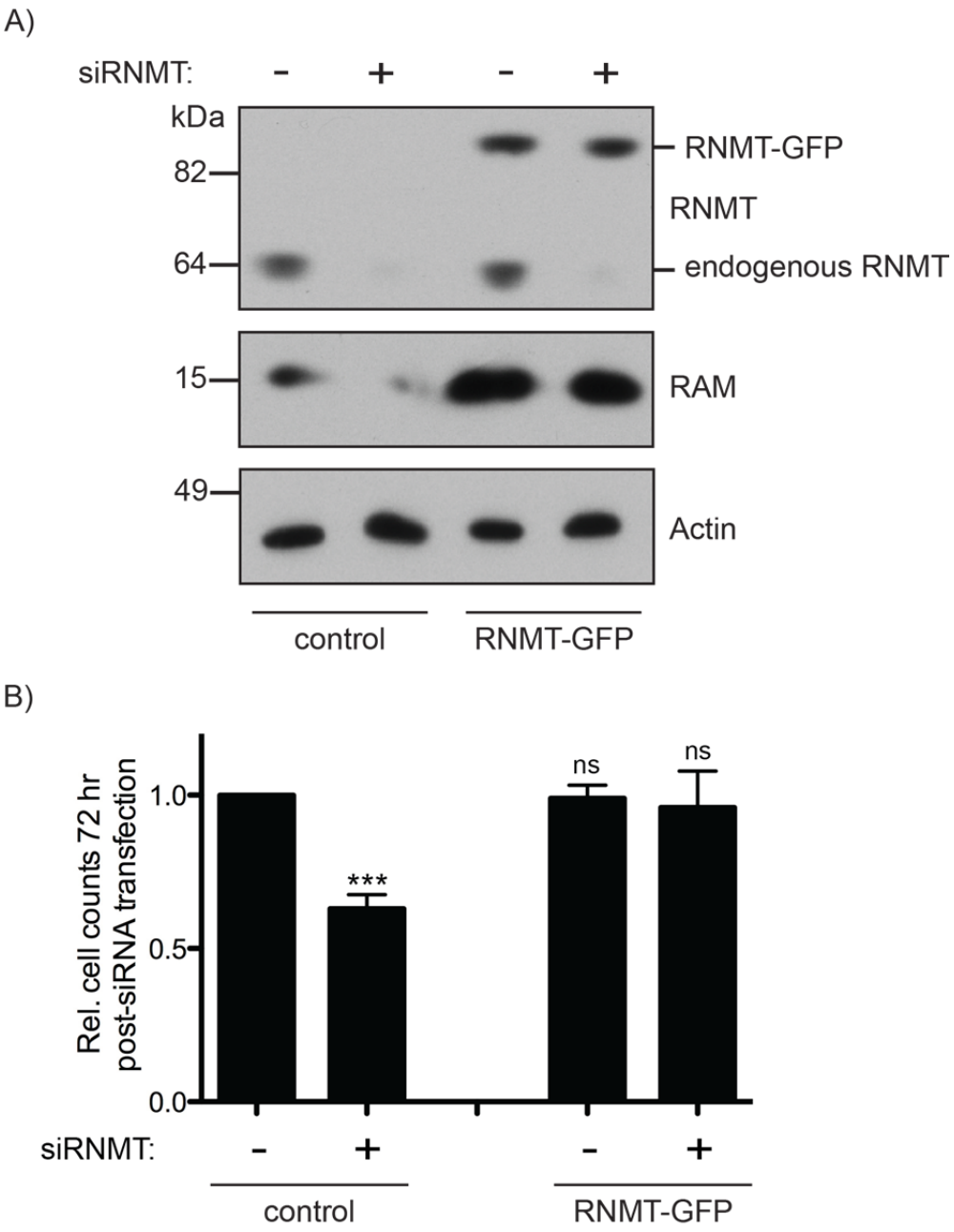


Figure 3.6 Exogenous expression of RNMT can rescue the proliferative defect induced by siRNA-mediated RNMT depletion

HCC-1806 cells stably expressing RNMT-GFP (RNMT-GFP), or vector control (control), were plated in equal numbers and transfected with siRNA targeting RNMT (siRNA RNMT 1) (+), or non-targeting control (-), for 72 hr. (**A**) Protein lysates were analysed by immunoblotting with the indicated antibodies. Result is representative of two independent biological replicates. Actin serves as loading control. (**B**) Cell counts relative to control (i.e. control cells transfected with non-targeting siRNA) and SD for three independent biological replicates are indicated. Statistical significance was assessed using ANOVA followed by Tukey's multiple comparison test using GraphPad Prism 5.0. ns $P > 0.05$, no significance difference compared with control cells treated with non-targeting siRNA; *** $P \leq 0.001$.

3.2.5 RNMT depletion induces apoptosis in a subset of breast cancer cells

Apoptosis is the process of programmed cell death and apoptosis could contribute to the reduced cell proliferation of cell lines “sensitive to RNMT depletion” (Evan and Vousden, 2001, Thompson, 1995). Interestingly, two studies have previously reported that a reduction in RNMT expression induces apoptosis in HeLa cells, a cervical cancer cell line (Chu and Shatkin, 2008, Gonatopoulos-Pournatzis et al., 2011). Therefore, I investigated the ability of RNMT depletion to induce apoptosis in breast cancer cells “sensitive” (MCF7 and HCC-1806) and “insensitive” (MDA-MB-231 and ZR.75.1) to RNMT depletion. A well-characterised indicator of apoptosis is the cleavage of the poly (adenosine diphosphate [ADP]-ribose) polymerase (PARP) protein from 112 kDa to 89 kDa. Thus, the induction of apoptosis in response to RNMT depletion was assessed by detecting PARP cleavage using immunoblot analysis. Cells were transfected with 3 independent siRNAs targeting RNMT, or a non-targeting siRNA, for 96 hr. The breast cancer cells were treated with straurosporine, a broad spectrum kinase inhibitor which is well-characterised inducer of apoptosis (Zhang et al., 2004). This served as a positive control for the immunoblot experiments. As shown in Figure 3.7 and Figure 3.8, for the four cell lines tested (MCF-7, HCC-1806, MDA-MB-231 and ZR.75.1), RNMT was efficiently depleted using each of the three independent RNMT siRNAs. Treatment of the cells with straurosporine induced the cleavage of PARP from 112 kDa to 89 kDa. The immunoblot experiments revealed that RNMT depletion, with all three independent siRNAs, induced PARP-cleavage in MCF7 and HCC-1806 cells, whereas it did not significantly induce PARP-cleavage in MDA-MB-231 and ZR.75.1 cells. These results show that RNMT depletion induces apoptosis in MCF7 and HCC-1806 cells, whereas it does not

significantly induce apoptosis in MDA-MB-231 and ZR.75.1 cells (Figure 3.7 and Figure 3.8). Taken together, these results show that RNMT depletion can induce apoptosis in a subset of breast cancer cell lines.

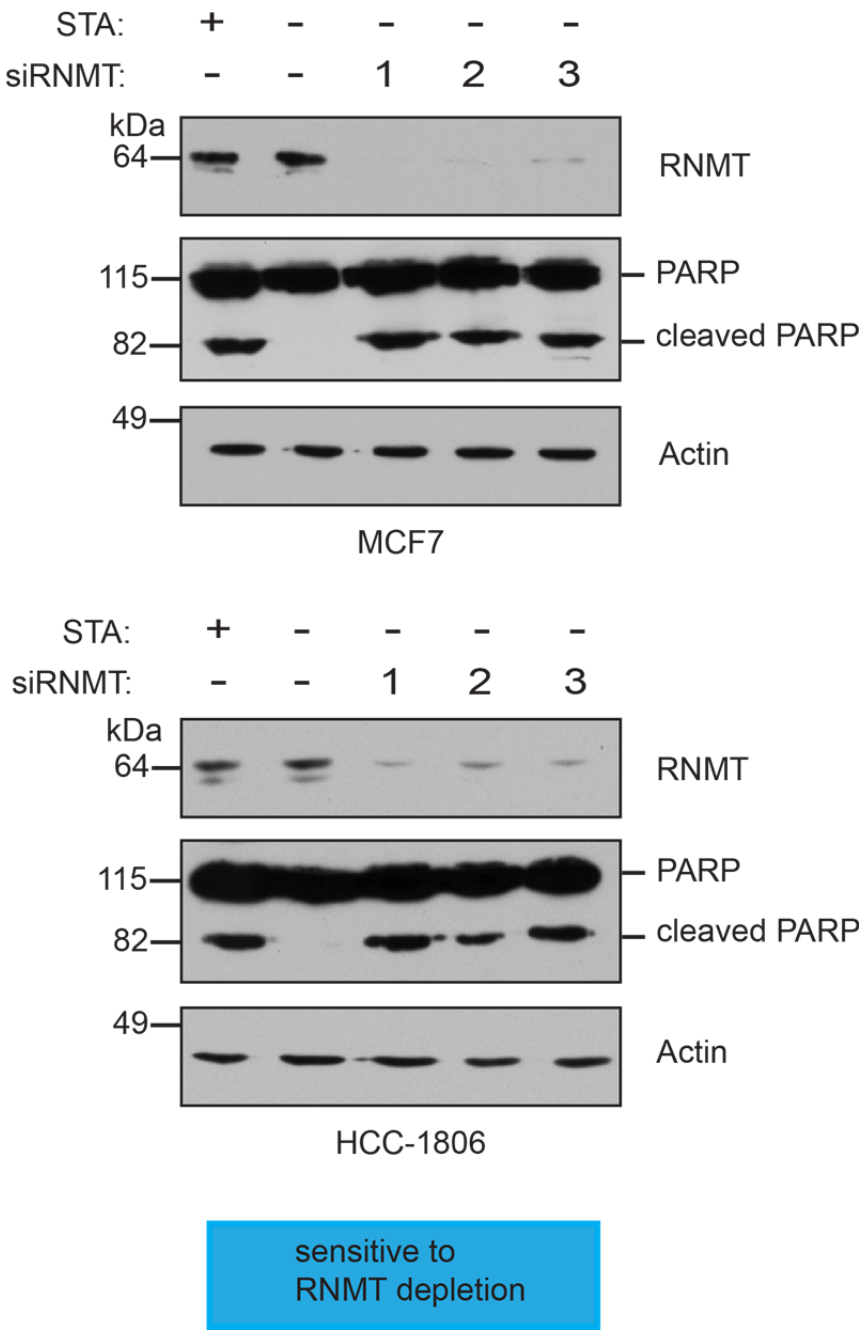


Figure 3.7 Apoptosis is detected in MCF7 and HCC-1806 cells depleted of RNMT

Cells were plated and transfected with 3 independent siRNAs targeting RNMT (1, 2, 3), or non-targeting control siRNA (-) for 96 hr. As a positive control for apoptosis, cells were treated with control siRNA for 72 hr followed with 1μM straurosporine (STA) (+) for 3 hr. Protein lysates were extracted and analysed by immunoblotting with the indicated antibodies. Actin serves as a loading control. The sensitivity of MCF7 and HCC-1806 cells to RNMT depletion is indicated.

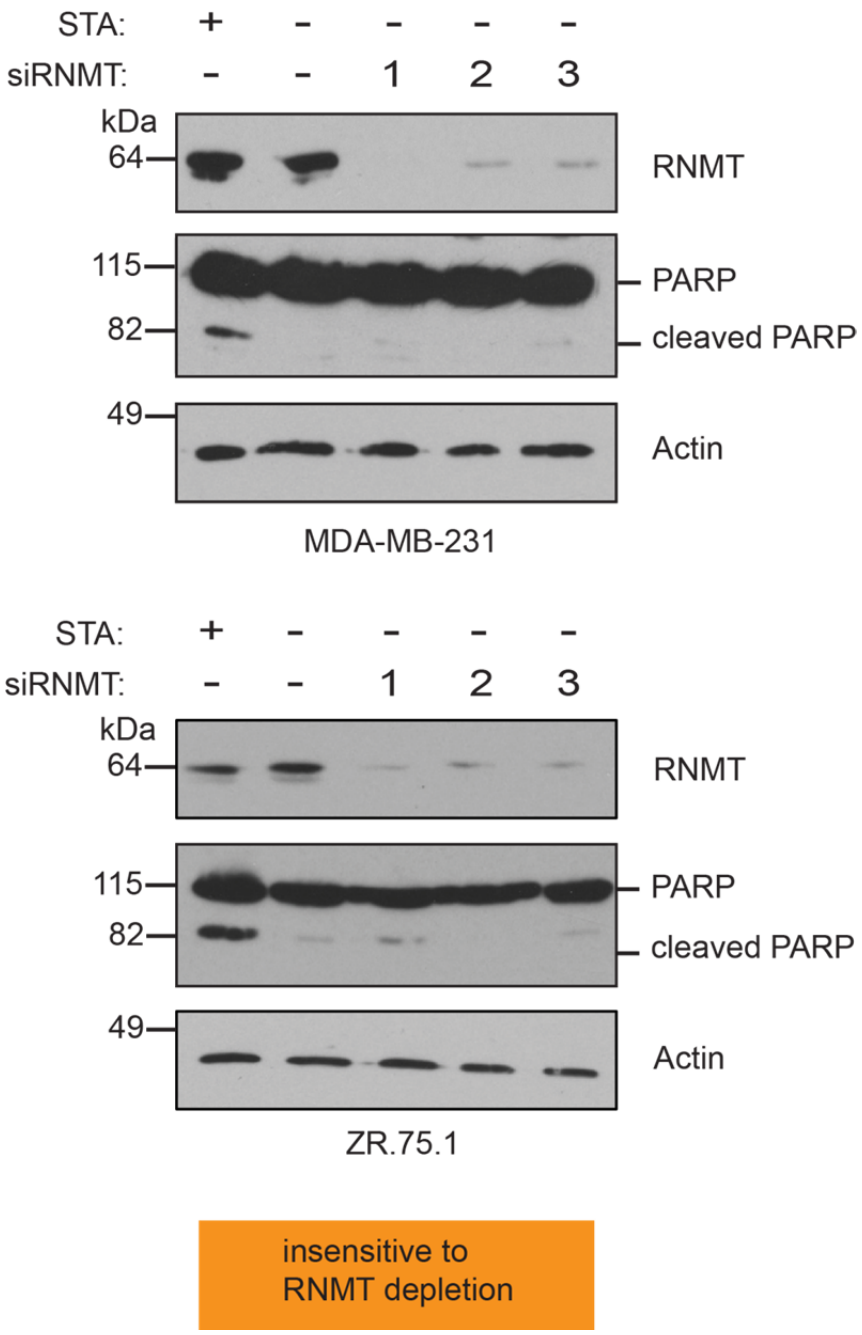


Figure 3.8 Apoptosis is not significantly detected in MDA-MB-231 and ZR.75.1 cells depleted of RNMT

Cells were plated and transfected with 3 independent siRNAs targeting RNMT (1, 2, 3), or non-targeting control siRNA (-) for 72 hr. As a positive control for apoptosis, cells were treated with control siRNA for 72 hr followed with 1μM straurosporine (STA) (+) for 3 hr. Protein extracts were analysed by immunoblotting with the indicated antibodies. Actin serves as a loading control. The sensitivity of MDA-MB-231 and ZR.75.1 cells to RNMT depletion is indicated.

3.3 Discussion

3.3.1 RNMT: a potential target in breast cancer?

Deregulated mRNA translation is a characteristic of cancer and there has been considerable effort into developing strategies to target mRNA translation initiation for cancer therapeutics (Blagden and Willis, 2011, Silvera et al., 2010). From yeast to humans, the mRNA methyl cap is essential for efficient gene expression and cell viability (Furuichi and Shatkin, 2000). In humans, RNMT catalyses the methylation of the guanosine cap at the N-7 position. RNMT expression is essential for cellular cap methylation and efficient gene expression (Cowling, 2009a, Cowling, 2009b). Emerging evidence suggests that there is strong biological rationale to explore RNMT as a therapeutic target in breast cancer (Cowling, 2009a, Fernandez-Sanchez et al., 2009a, Cole and Cowling, 2009). An effective therapeutic target should reduce the proliferation of cancerous cells, but cause limited toxicity to non-cancerous cells.

RNMT has previously been shown to be rate-limiting for efficient cell proliferation (Chu and Shatkin, 2008, Aregger and Cowling, 2013, Gonatopoulos-Pournatzis et al., 2011). However, there are no published data exploring whether inhibition of RNMT supresses the proliferation of all cells, or has selectivity for cancer cells. Therefore, in this thesis I set out to explore the suitability of RNMT as a therapeutic target in breast cancer. The data presented in this chapter has demonstrated for the first time that that RNMT depletion selectively inhibits the proliferation of breast cancer cells, in comparison to immortalised mammary epithelial cells. These data strongly suggest that RNMT should be explored as a therapeutic target in breast cancer. My findings add to the increasing body of data that has shown that targeting mRNA translation

initiation can selectively inhibit the growth of cancer cells (Silvera et al., 2010, Blagden and Willis, 2011, Graff et al., 2007, Moerke et al., 2007).

3.3.2 RNMT depletion selectively inhibits the proliferation of a subset of breast cancer cell lines

When targeting an essential process such as mRNA methyl cap synthesis, a major question is whether it will be significantly toxic to non-cancerous cells. Results in this chapter have shown that RNMT depletion does not significant affect the proliferation of immortalised mammary epithelial cells (IMECs). This suggests that non-transformed cells are less dependent on RNMT expression in comparison to certain cancer cells. Since detectable levels of RNMT expression remained in response to siRNA transfection, it is not possible to conclude that IMECs do not require RNMT for proliferation. It is likely that the low levels of RNMT expression that remained are enough to maintain cell proliferation. In future, it would be of interest to explore the effects of RNMT depletion in a different breast-derived non-transformed cell line, such as MCF10A, to fully establish the validity of the results.

These observations demonstrate that a subset of breast cancer cells are significantly more dependent on RNMT expression for proliferation, in comparison to IMECs. Since three independent siRNAs targeting RNMT produced a similar effect and exogenous expression of siRNA-resistant RNMT rescued the impaired proliferation of RNMT depleted cells, it is likely that the observed proliferative defects of RNMT depleted cells is not simply due to siRNA off-target effects. Although our laboratory is currently screening for specific RNMT inhibitors in collaboration with the Drug Discovery Unit (College

of Life Sciences, University of Dundee), at present there are no specific inhibitors for RNMT. To establish the validity of my findings, it would be desirable to investigate the effects of an RNMT inhibitor on the breast cancer cell panel.

It has been reported that RNMT exists in a complex with RAM (RNMT-activating mini protein). RAM enhances RNMT activity *in vitro* up to 5-fold and RAM expression is essential for cellular cap methylation and efficient gene expression (Gonatopoulos-Pournatzis et al., 2011). It has previously been reported that the expression of RAM protein is dependent on RNMT expression, and vice a versa (Gonatopoulos-Pournatzis et al., 2011). Consistent with this, for all the cell lines tested in this present study, a reduction in RNMT expression lead to a corresponding decrease in RAM protein expression. Thus, it is possible that RAM loss contributes to the reduced proliferative capacity of the "RNMT depletion sensitive" breast cancer cells.

It is possible that the differential dependency of breast cancer cells on RNMT for proliferation is simply due to differences in RNMT depletion levels between cells "sensitive to RNMT depletion" and those cells "insensitive to RNMT depletion". Western blot analysis, which can only be considered semi-quantitative, of two representative cell lines from each group revealed that RNMT depletion levels were comparable in the two groups of cells.

3.3.3 RNMT depletion induces apoptosis in a subset of breast cancer cells

The observed reduction in cell number in RNMT depleted breast cancer cells could result from either a reduction in cell growth, or an increase in the rate of apoptosis (Evan and Vousden, 2001, Thompson, 1995). For the breast cancer cell lines “sensitive to RNMT depletion”, I observed that there was a substantial increase in the number of floating cells in the petri dish in response to RNMT depletion. Two previous studies have shown that RNMT depletion induces apoptosis in HeLa cells, a cervical cancer cell line (Chu and Shatkin, 2008, Gonatopoulos-Pournatzis et al., 2011). Measuring PARP cleavage using immunoblot analysis confirmed that RNMT depletion induces apoptosis in breast cancer cells “sensitive to RNMT depletion” but does not in those “insensitive to RNMT depletion”. These results suggest that apoptosis contributes to the reduction in cell number of breast cancer cells “sensitive to RNMT depletion”. The ability of RNMT to induce apoptosis in a subset of breast cancer cells provides evidence to suggest that targeting RNMT could be a suitable therapeutic target in a subset of breast cancer. However, in the future, it will be important to investigate whether RNMT depletion selectively induces apoptosis in breast cancer cells, in comparison to IMECs.

A number of methods exist to detect apoptotic cells and it would be important to use several independent approaches to confirm the validity of my findings. For example, activated caspase-3, which an indicator of apoptosis could be measured using enzyme-linked immunosorbent assay (ELISA), or fluorescence activated cell sorting (FACS). Moreover, DNA fragmentation, which occurs during apoptosis, could be measured using terminal deoxynucleotidyl transferase nick end labelling (TUNEL).

In addition, it would be important to investigate the mechanisms by which RNMT depletion induces apoptosis in breast cancer cells. The mRNA methyl cap is essential for efficient cap-dependent translation and RNMT expression is rate-limiting for a subset of mRNAs (Cowling, 2009a). The balance of cellular pro- and anti-apoptotic proteins determines cell fate. It is possible that RNMT depletion inhibits the expression of certain anti-apoptotic proteins, but does not significantly affect the expression of pro-apoptotic proteins. If so, RNMT depletion may induce apoptosis by tipping the balance of cellular pro- and anti-apoptotic proteins in favour of pro-apoptotic ones. Interestingly, several proteins which induce apoptosis, such as c-Myc and p53, are translated via a cap-independent mechanism and expression of these proteins would likely be maintained in response to RNMT depletion (Hoffman and Liebermann, 1998, Spriggs et al., 2005, Yang et al., 2006, Ray et al., 2006, Liwak et al., 2012). It would therefore be of interest to investigate whether c-Myc and p53 are mediating the apoptotic effects observed in RNMT depleted cells.

The reduced proliferation observed in cells depleted of RNMT may be caused completely by apoptosis, or it may be caused by the combined effect of apoptosis and growth arrest. Z-VAD.FMK is a caspase inhibitor that inhibits cellular apoptosis (Slee et al., 1996). To investigate whether the reduced proliferation of cells is completely dependent on apoptosis, the combined effect of Z-VAD.FMK and siRNA RNMT on the proliferation of breast cancer cells should be explored. If the above experiments indicate that growth arrest contributes to the reduced proliferation of RNMT depleted cells, it would be of interest to explore whether RNMT depletion affects cell cycle progression. This could be assessed by monitoring cellular DNA content using DNA staining followed by FACS.

3.3.4 Potential mechanisms of RNMT dependency in breast cancer cells

The methyl cap is essential for efficient mRNA translation and it has been shown to promote several steps in gene expression (Furuichi and Shatkin, 2000). It is likely that the breast cancer cells which are “sensitive to RNMT depletion” have an enhanced dependency on the methyl cap for survival. Since the methyl cap is essential for the translation of most mRNAs, inhibition of cap methylation has the potential to inhibit the actions of any oncogene that deregulates gene expression. Thus, targeting RNMT could be effective in a wide range of tumours driven by different oncogenes. However, the molecular mechanisms that render breast cancer cells dependent on RNMT expression for proliferation are unclear, and this is investigated in chapter 4 and chapter 5.

eIF4E binds the mRNA methyl cap and eIF4E expression is rate-limiting for mRNA translation. There is a large body of data that suggests eIF4E is an effective therapeutic target in cancer (De Benedetti and Graff, 2004). Since the methylation of the guanosine cap is essential for efficient binding of eIF4E to the methyl cap (Niedzwiecka et al., 2002, von der Haar et al., 2004), it is possible that targeting RNMT could inhibit eIF4E function in cancer cells. It is possible that the breast cancer cells which are “sensitive to RNMT depletion” may have an enhanced dependency on eIF4E activity. This issue should be investigated further in future experiments.

3.3.5 Summary

Overall, the results presented in this chapter show that a subset of breast cancer cells have an enhanced dependency on RNMT for survival in comparison to immortalised mammary epithelial cells. These findings strongly suggest that RNMT should be perused as a therapeutic target in breast cancer. Furthermore, my findings have demonstrated for the first time that targeting methyl cap synthesis can selectively inhibit the proliferation of cancer cells.

Chapter 4: Investigating cellular sensitivity to RNMT depletion

4.1 Introduction

The results in chapter 3 demonstrated that RNMT depletion impairs the proliferation of a subset of breast cancer cell lines, but does not significantly affect the proliferation of immortalised mammary epithelial cells (IMECs).

Many cancer therapies aim to exploit the fact that cancer cells exhibit enhanced dependence on a signalling pathway by targeting that pathway. Since a subset of breast cancer cells are more dependent on RNMT for survival, in comparison to IMECs, targeting RNMT may be an effective anti-cancer strategy in a subset of breast cancers. However, it is important that we understand the molecular mechanisms that render breast cancer cells dependent upon RNMT for proliferation and survival.

Therefore, I decided to explore the factors that determine cellular sensitivity to RNMT depletion. In this chapter, I investigate whether there is a correlation between various cellular parameters and cellular sensitivity to RNMT depletion in the cell line panel that was described in the previous chapter.

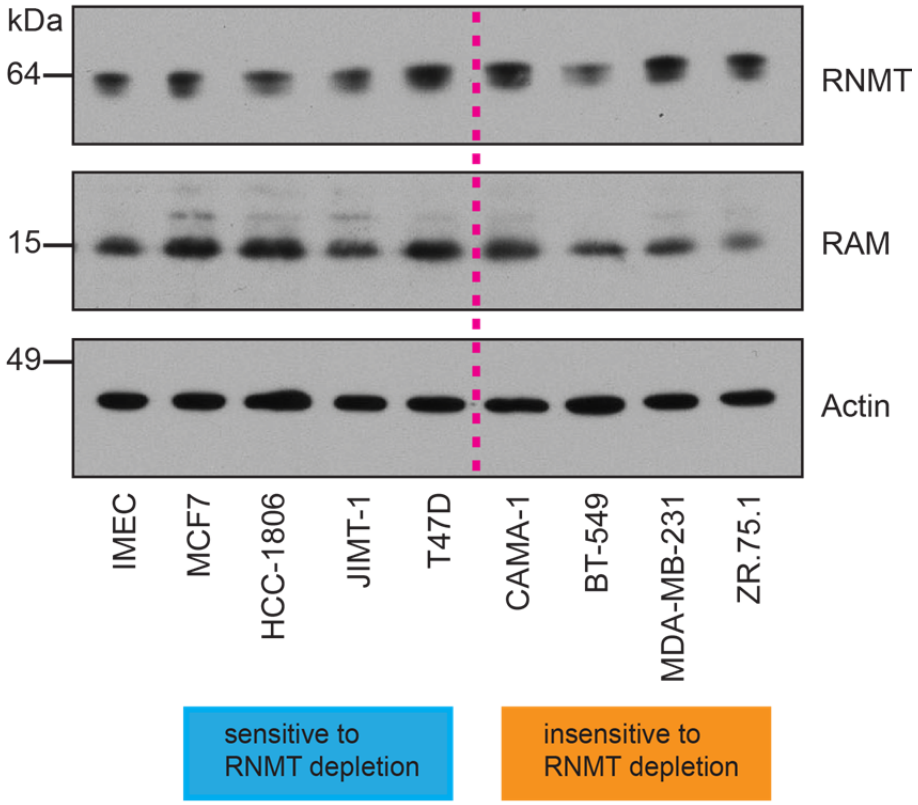
4.2 Results

4.2.1 RNMT protein is expressed at comparable levels across the breast cancer cell line panel

The observation that experimental overexpression of RNMT is sufficient to transform mammary epithelial cells indicates that elevated RNMT expression could have the potential to contribute to breast cancer. This raises the possibility that breast cancer cells with elevated RNMT expression may exhibit an enhanced dependency on RNMT for survival. A search of ONCOMINE (a web-based microarray database) revealed that RNMT expression is not higher in breast cancer tissue in comparison to non-cancerous tissue. Moreover, no studies to date have examined RNMT protein expression in breast cancer. In order to test whether there was a relationship between RNMT expression and cellular sensitivity to RNMT depletion, I compared RNMT protein levels in the previously used eight breast cancer cell lines and in IMECs using immunoblot analysis with anti-RNMT and anti-RAM antibodies. As shown in Figure 4.1, RNMT expression levels were fairly comparable across all cell lines tested. As mentioned in the previous chapter, RNMT and RAM protein expression are co-dependent (Gonatopoulos-Pournatzis et al., 2011). Consistent with an equivalent expression of RNMT, the expression of RAM protein was also fairly equivalent across the cell lines tested (Figure 4.1). However, it should be noted that between multiple biological replicates, I observed slight fluctuations in RNMT and RAM levels, which made it difficult to confidently conclude that RNMT and RAM were equivalently expressed across the cell lines panel. In an attempt to address this issue, I quantified the intensity of RNMT and RAM immunoblot bands from four independent biological replicates using Image J software (Figure 4.1B).This analysis revealed that there was no significant

difference in the protein expression levels of either RNMT or RAM across the cell panel ($P > 0.05$, one-way ANOVA). In order to help compare RNMT expression levels between the breast cancer cell lines “sensitive” and “insensitive” to RNMT depletion, the breast cancer cells in Figure 4.1, are arranged in order of decreasing sensitivity to RNMT depletion (from left to right). It is important point out that this order is used for several subsequent figures in this chapter (Figure 4.4, Figure 4.5, Figure 4.6, Figure 4.7 and Figure 4.8). Overall these results show that expression of RNMT and RAM is not higher in breast cancer cells, in comparison to non-transformed mammary epithelial cells. Furthermore, RNMT and RAM protein is not differentially expressed between breast cancer cells “sensitive” (MCF7, HCC-1806, JIMT-1 and T47D) and “insensitive” (CAMA-1, BT-549, MDA-MB-231 and ZR.75.1) to RNMT depletion (Figure 4.1). These results indicate that RNMT protein expression levels do not correlate with cellular sensitivity to RNMT depletion.

A)


kDa
64
15
49
RNMT
RAM
Actin
IMEC
MCF7
HCC-1806
JIMT-1
T47D
CAMA-1
BT-549
MDA-MB-231
ZR.75.1
sensitive to RNMT depletion
insensitive to RNMT depletion

B)

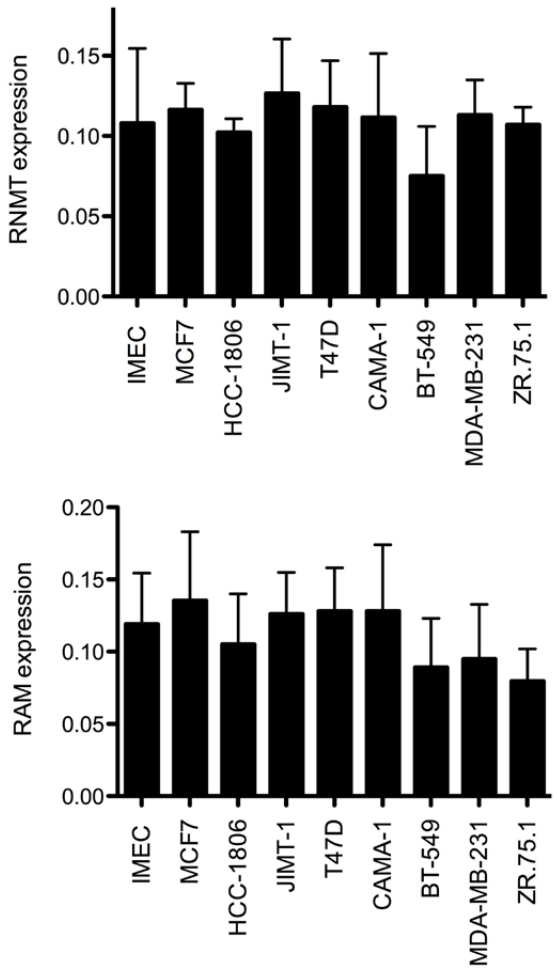

RNMT expression
0.15
0.10
0.05
0.00
IMEC
MCF7
HCC-1806
JIMT-1
T47D
CAMA-1
BT-549
MDA-MB-231
ZR.75.1
RAM expression
0.20
0.15
0.10
0.05
0.00
IMEC
MCF7
HCC-1806
JIMT-1
T47D
CAMA-1
BT-549
MDA-MB-231
ZR.75.1

Figure 4.1 RNMT and RAM expression levels are similar across the cell line panel

A) Protein lysates were analysed by immunoblotting with the indicated antibodies. Actin serves as a loading control. Result is representative of four biological replicates. The sensitivity of the breast cancer cells to RNMT depletion are indicated. **B**) RNMT and RAM immunoblot band intensities were quantified using Image J software. Bar chart depicts the mean band intensity quantification. SD for four independent biological replicates is indicated. Statistical significance was assessed by ANOVA using GraphPad Prism 5. For both RNMT and RAM expression, there was no significant difference in the mean band intensities across the cell lines ($P > 0.05$).

4.2.2 Cellular mRNA cap methyltransferase activity levels does not correlate with cellular sensitivity to RNMT depletion

Cellular mRNA cap methyltransferase activity is dependent on RNMT expression (Cowling, 2009a). However, previous experiments in our laboratory have demonstrated that RNMT enzymatic (cap methyltransferase) activity can be regulated by phosphorylation (Aregger, unpublished). Therefore, despite RNMT expression levels being similar across the cell panel (Figure 4.1) the enzymatic activity could be different and may correlate with cellular sensitivity to RNMT depletion. To address this, I next sought to establish the mRNA cap methyltransferase activity levels across the cell panel. Cellular cap methyltransferase activity can be determined using an *in vitro* assay, which was established by the Shuman lab (Mao et al., 1995). This assay was performed as previously described in section 2.2.27 (Figure 4.2). To be able to accurately measure cap methyltransferase activity across the cell panel, it was important to first determine the linear range of the assay for each cell line. Therefore, protein lysates were extracted from the cell panel and the *in vitro* cap methyltransferase assay was performed using a titration of protein lysates from 0.25 µg to 1 µg. For all the cell lines tested (IMEC, MCF7, HCC-1806, JIMT-1, T47D, BT-549, CAMA-1, MDA-MB-231 and ZR.75.1), the assay was found to be linear between 0.25 µg to 1 µg of protein lysate (Figure 4.3). Therefore, I decided to use 0.5 µg of protein lysates for each reaction to compare the RNMT activity levels across the cell panel. As shown in Figure 4.4, it was observed that two of the breast cancer cell lines (HCC-1806 and CAMA-1) have significantly lower RNMT activity levels, compared to IMECs ($P \leq 0.5$, ANOVA, followed by Dunnett's multiple comparison test). Whereas, the RNMT activity levels between the remaining six breast cancer cell lines

(MCF7, JIMT-1, T47D, BT-549, MDA-MB-231 and ZR.75.1) and IMECs are not significantly different ($P > 0.05$, ANOVA followed by Dunnett's multiple comparison test). It should be noted that IMECs and MCF7 cells have the highest RNMT activity levels of all the cell lines tested (Figure 4.4). The above results show that, except for MCF7 cells, breast cancer cells generally have lower RNMT activity levels compared to IMECs. Furthermore, as shown in Figure 4.4, there is no significant difference in the cap methyltransferase levels between the breast cancer cells "sensitive" (MCF7, HCC-1806, JIMT-1, T47D) and "insensitive" (CAMA-1, MDA-MB-231, ZR.75.1 and BT-549) to RNMT depletion. On the basis of these findings, I concluded that cap methyltransferase activity levels of the cell panel do not correlate with cellular sensitivity to RNMT depletion.

Although it was shown that RNMT depletion levels are comparable in the breast cancer cells "sensitive" and "insensitive" to RNMT depletion (Figure 3.5), it was possible that there could be differences in the cap methyltransferase levels. Therefore, I next sought to measure cap methyltransferase levels in the RNMT depleted cell line panel. Cells were transfected with siRNA targeting RNMT, or a non-targeting siRNA, for 48 hr and the *in vitro* cap methyltransferase assay was performed on cell lysates. This revealed that there is approximately a two-fold decrease in cap methyltransferase levels in all the cell lines (Figure 4.5). Thus, it can be concluded that cap methyltransferase levels are equivalently depleted across the cell line panel using siRNA-mediated RNMT depletion.

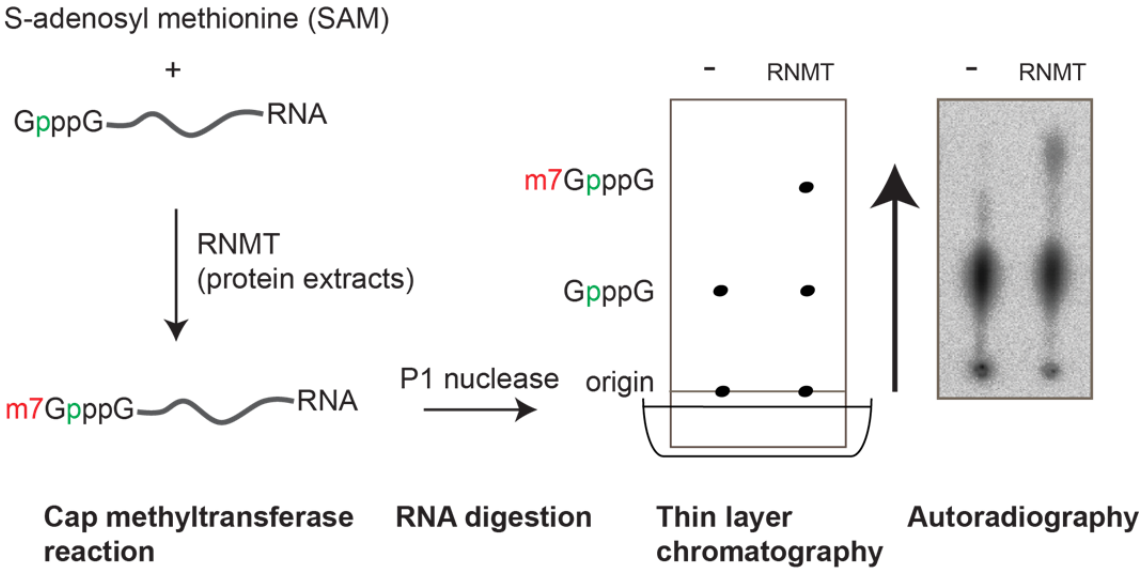
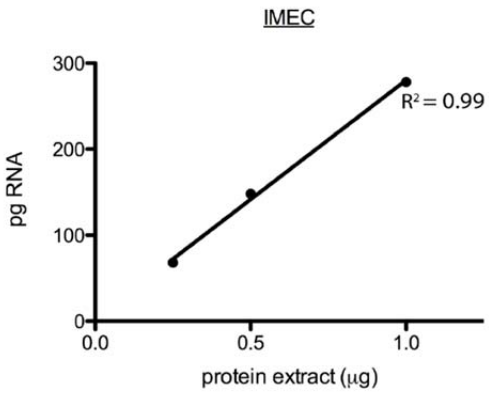
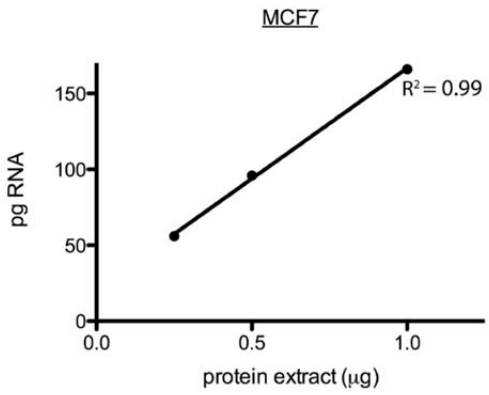
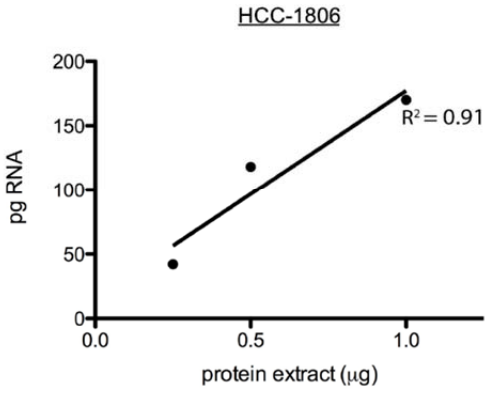


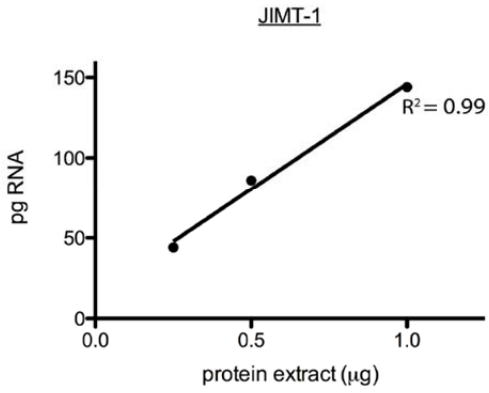
Figure 4.2 Schematic of the *in vitro* cap methyltransferase assay

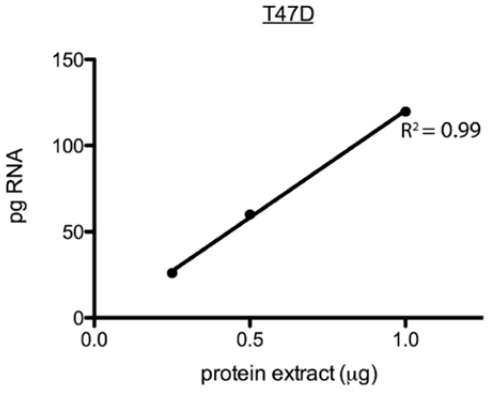
The schematic illustrates the major steps of the assay and a typical autoradiography exposure is shown. Negative control without protein (-) and protein extract (RNMT) is indicated. To determine cellular cap methyltransferase levels, protein extracts were incubated with 32P-labelled capped *in vitro* transcribed RNA and the methyl donor S-adenosyl methionine (SAM). The resultant RNA was subsequently incubated with P1 nuclease to release the cap structures (GpppG and m7GpppG), which were then resolved by thin layer chromatography (TLC.) The conversion of cap (GpppG) to methyl cap (m7GpppG) was visualised using autoradiography and quantified.

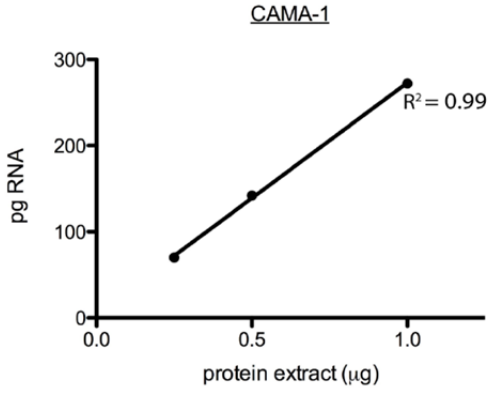

IMEC
pg RNA
300
200
100
0
0.0
0.5
1.0
protein extract (μg)
R² = 0.99


MCF7
pg RNA
150
100
50
0
0.0
0.5
1.0
protein extract (μg)
R² = 0.99


HCC-1806
pg RNA
200
150
100
50
0
0.0
0.5
1.0
protein extract (μg)
R² = 0.91


JIMT-1
pg RNA
150
100
50
0
0.0
0.5
1.0
protein extract (μg)
R² = 0.99


T47D
pg RNA
150
100
50
0
0.0
0.5
1.0
protein extract (μg)
R² = 0.99


CAMA-1
pg RNA
300
200
100
0
0.0
0.5
1.0
protein extract (μg)
R² = 0.99

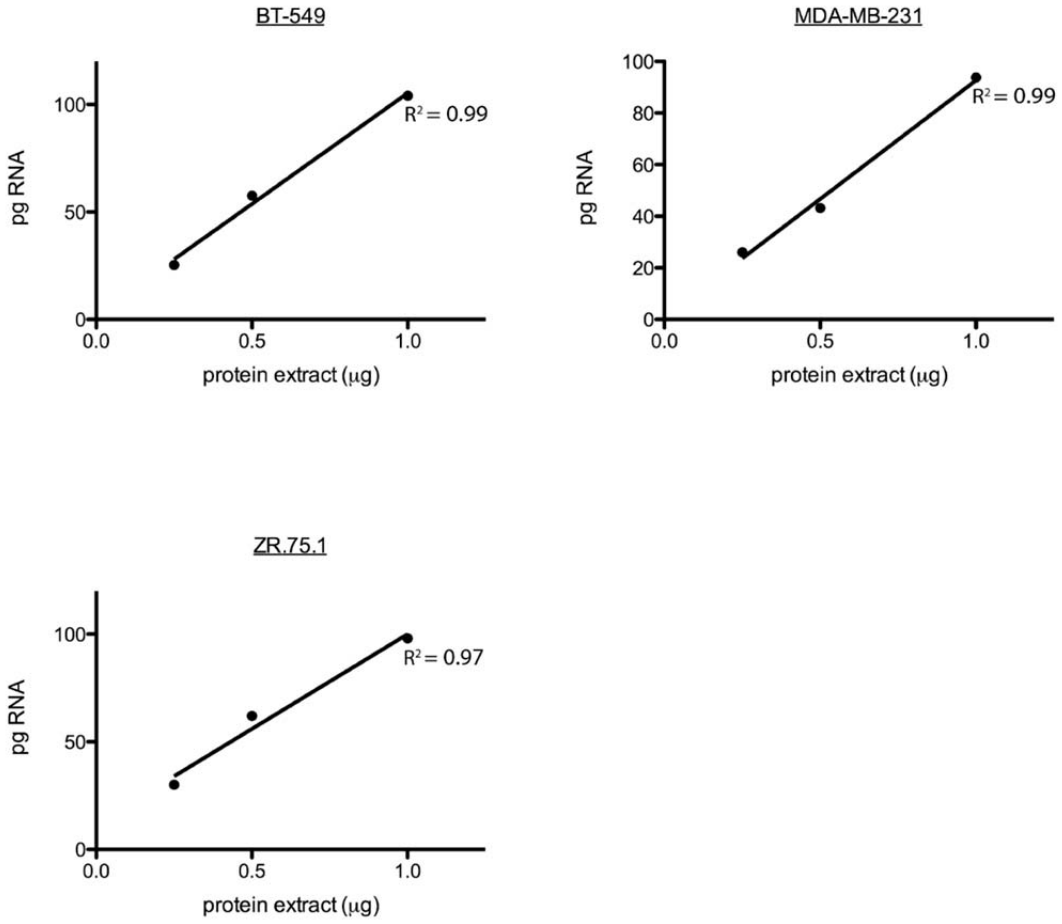


Figure 4.3 Optimisation of the *in vitro* cap methyltransferase assay for use with the cell panel

Protein lysates were extracted from cells and the *in vitro* cap methyltransferase assay was performed using 0.25, 0.5 and 1 µg of protein lysates, or without lysates as a negative control, in the presence of *in vitro* transcribed [32-P]-capped RNA and S-adenosyl-methionine. GpppG and m7GpppG were resolved using thin layer chromatography. [32-P] signal was detected using a phosphorimager and quantified using AIDA Image analyser. Each graph depicts the linear regression for each cell line between protein extract (µg) and the concentration of methyl capped RNA (pg) for one biological replicate carried out in duplicate. The linear regression coefficient (R^2) was determined using GraphPad Prism 5.0. and is indicated on each graph.

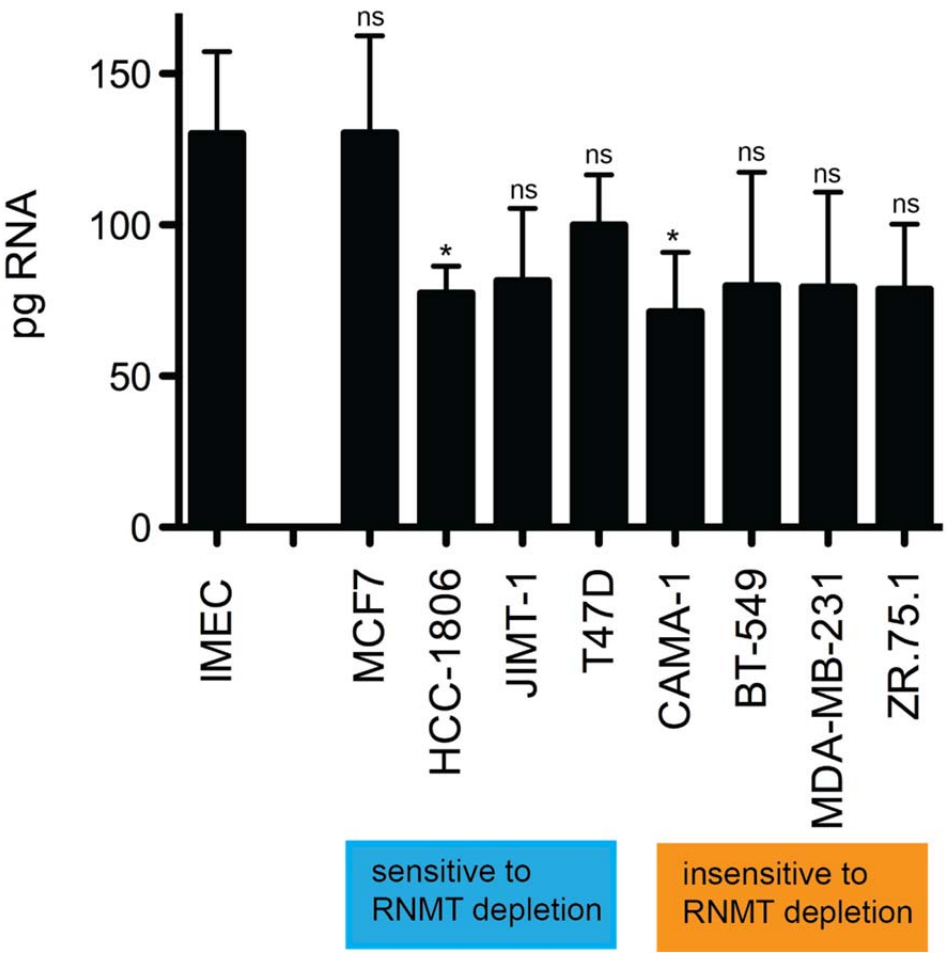


Figure 4.4 mRNA cap methyltransferase activity levels in IMECs and breast cancer cells do not correlate with cellular sensitivity to RNMT depletion

Protein lysates were extracted from cells and the *in vitro* cap methyltransferase assay was performed using 0.5 µg of total protein, or without lysate as a negative control, in the presence of *in vitro* transcribed [32P]-capped RNA and S-adenosyl-methionine. GpppG and m7GpppG were resolved using thin layer chromatography. [32P] signal was detected using a phosphorimager and quantified using AIDA Image analyser. Bar chart depicts the mean concentration of m7GpppG RNA (pg). SD for four biological replicates is indicated. Statistical significance was assessed using ANOVA followed by Dunnett's multiple comparison test using GraphPad Prism 5.0. ns, $P > 0.05$ no significant difference compared to IMEC,* $P \leq 0.05$.

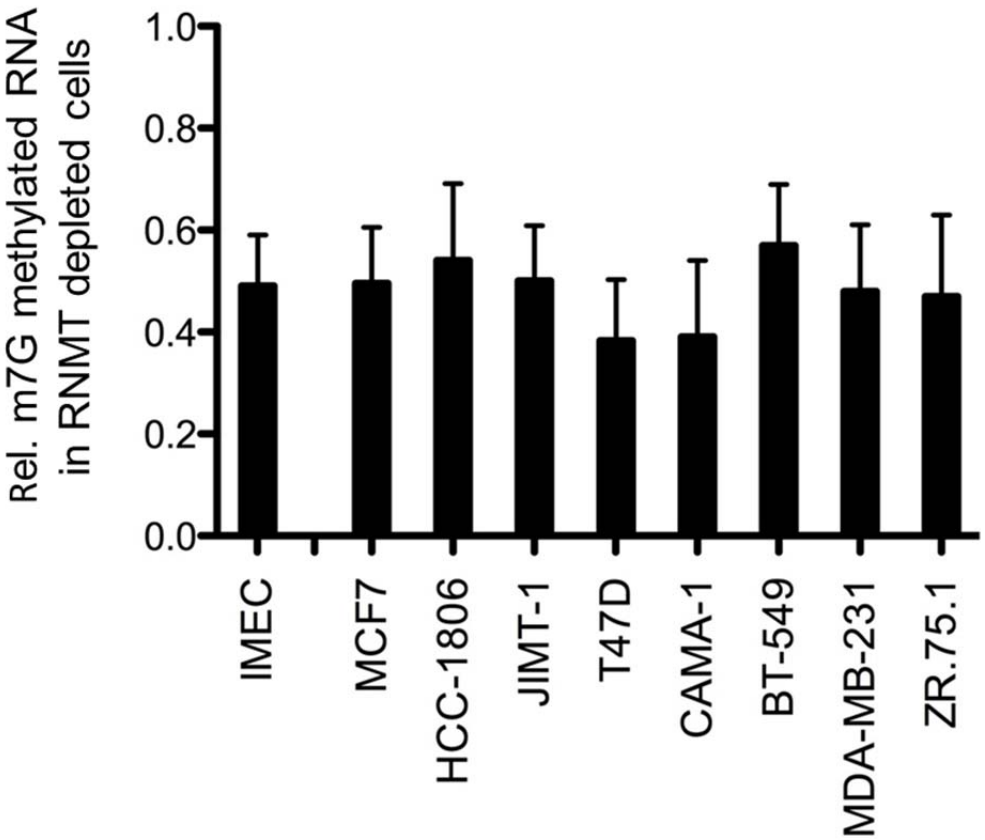


Figure 4.5 mRNA cap methyltransferase activity levels are equivalently depleted in IMECs and breast cancer cells using siRNA-mediated RNMT depletion

Cells were transfected with siRNA targeting RNMT or a non-targeting siRNA, for 48 hr. The *in vitro* cap methyltransferase assay was performed using 1 µg of protein lysate, or without lysate as a negative control, in the presence of *in vitro* transcribed [32P]-capped RNA and S-adenosyl-methionine. GpppG and m7GpppG were resolved using thin layer chromatography. [32P] signal was detected using a phosphorimager and quantified using AIDA Image analyser. Bar chart depicts the mean concentration of m7GpppG RNA (pg) in RNMT depleted cells relative to cells transfected with non-targeting siRNA. SD for three biological replicates is indicated. Statistical significance was assessed by ANOVA using GraphPad Prism 5. There was no significant difference in the cap methyltransferase activity levels across the cell line panel ($P > 0.05$).

4.2.3 Global protein synthesis rates do not correlate with cellular sensitivity to RNMT depletion

The increased proliferation of cancer cells is often dependent upon upregulated mRNA translation (Blagden and Willis, 2011, Johnson et al., 1976). The efficient translation of most mRNAs is dependent upon the methylation of the mRNA methyl cap (Muthukrishnan et al., 1975, Schwer et al., 2000, Drummond et al., 1985). This raises the question whether cells with heightened protein synthesis rates exhibit an enhanced dependency on RNMT for proliferation. To test whether there was a relationship between the rate of global protein synthesis and cellular sensitivity to RNMT depletion, I measured the incorporation of 35S-labelled amino acids into proteins in the cell panel. As shown in Figure 4.6, compared to IMECs, 35S incorporation was significantly higher in four breast cancer cell lines (JIMT-1: $P \leq 0.001$; HCC-1806: $P \leq 0.001$; ZR.75.1: $P \leq 0.001$; CAMA-1: $P \leq 0.5$; ANOVA followed by Dunnett's multiple comparison test), whereas the 35S incorporation between IMECs and the remaining four breast cancer cell lines was not significantly different (MCF7, T47D, BT-549 and MDA-MB-231: $P > 0.5$; ANOVA, followed by Dunnett's multiple comparison test; Figure 4.6). Overall these experiments show that a subset of breast cancer cells (HCC-1806, JIMT-1, CAMA-1 and ZR.75.1) exhibit an enhanced rate of global protein synthesis compared IMECs. However, this includes breast cancer cell lines which are "sensitive" (HCC-1806 and JIMT-1) as well as those "insensitive" (CAMA-1 and ZR.75.1) to RNMT depletion. The above findings suggest that an enhanced rate of protein synthesis alone does not confer cellular sensitivity to RNMT depletion.

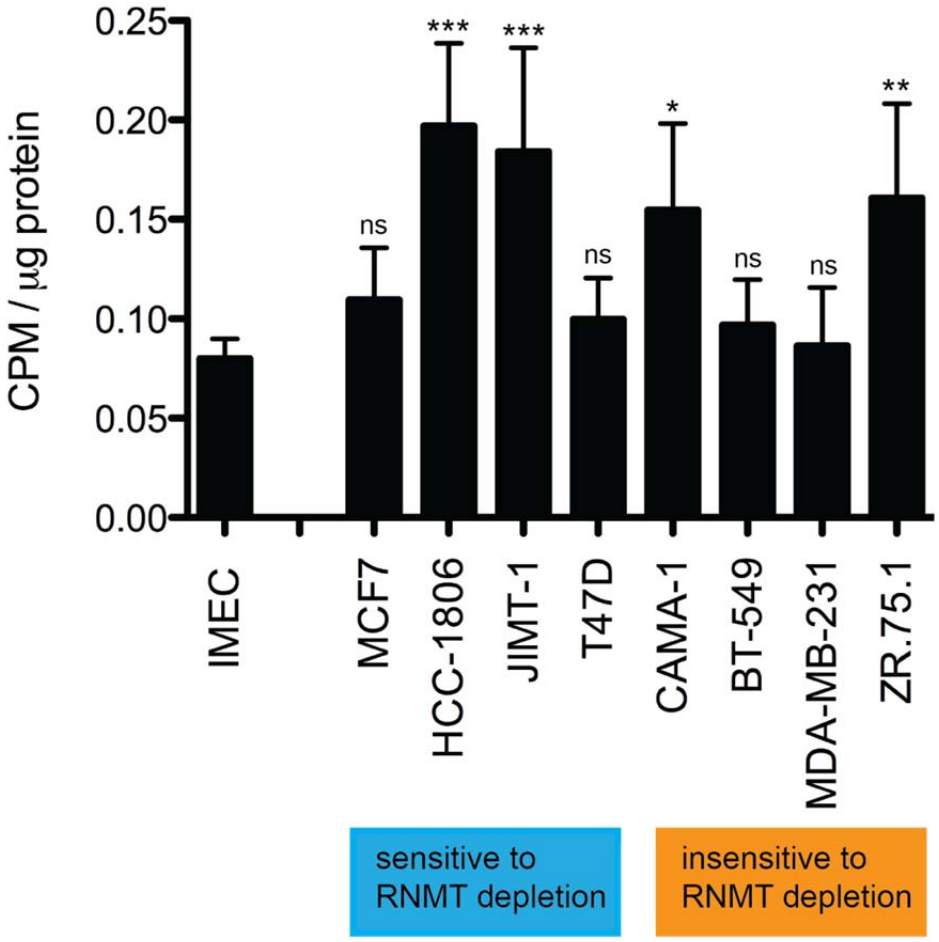


Figure 4.6 Basal rate of global protein synthesis in IMECs and breast cancer cells does not correlate with cellular sensitivity to RNMT depletion

Cells were incubated with media containing [35S]-labelled Met/Cys. [35S]-signal was detected using a phosphorimager and quantified using AIDA Image analyser. For each biological replicate, samples were labelled at duplicate and the bar chart depicts the mean CPM per µg of protein. SD for three independent biological replicates is shown. Statistical significance was assessed using ANOVA followed by Dunnett's multiple comparison test using GraphPad Prism 5.0. ns $P > 0.05$, no significant difference compared to IMEC; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$. The sensitivity of breast cancer cells to RNMT depletion is indicated.

4.2.4 RNMT depletion does not significantly affect the rate of global protein synthesis

The mRNA methyl cap is essential for efficient protein translation and RNMT expression has been shown to be rate-limiting for the translation of a subset of proteins (Cowling, 2009a). Therefore, the differential sensitivity of cells to RNMT depletion could be a due to a differential decrease in global protein synthesis. To test this hypothesis, IMECs and five breast cancer cell lines (MCF7, HCC-1806, JIMT-1, MDA-MB-231 and BT-549) were transfected with siRNA targeting RNMT, or non-targeting siRNA, and 40-48hr post-siRNA transfection and the global rate of protein synthesis was measured using 35S-incorporation into proteins. This revealed that for MCF7 cells, RNMT depletion modestly reduced 35S incorporation by 25% (Figure 4.6). However, the effect of RNMT depletion on 35S incorporation between IMECs and all five breast cancer cell lines was not found to be significant (MCF7, HCC-1806, JIMT-1, MDA-MB-231 and BT-549) ($P < 0.05$; ANOVA, followed by Dunnett's multiple comparison test). These results indicate that RNMT depletion does not significantly impact the rate of global protein synthesis in non-transformed mammary epithelial cells (IMEC) and in breast cancer cells "sensitive" (MCF7, HCC-1806 and JIMT-1) or "insensitive" (BT-549 and MDA-MB-231) to RNMT depletion. Taken together these results suggest that cellular sensitivity to RNMT depletion is not due to a differential reduction in global protein synthesis.

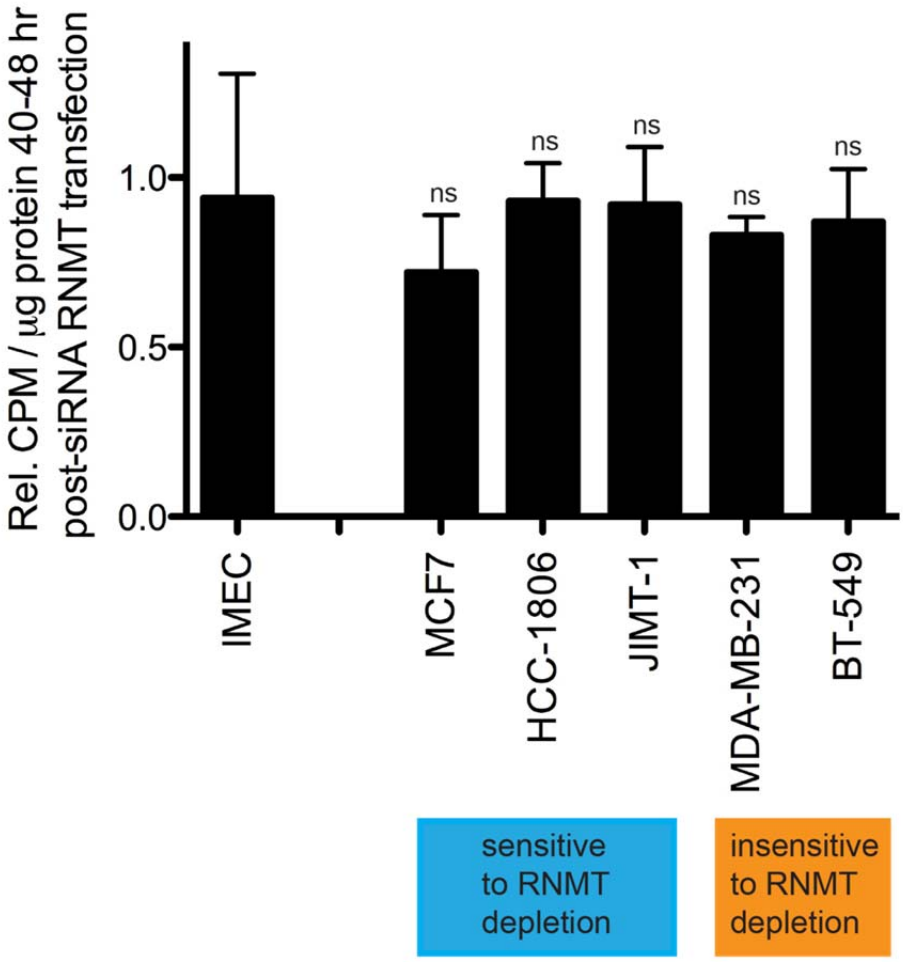


Figure 4.7 RNMT depletion does not significantly affect the rate of protein synthesis in IMECs and breast cancer cells

Cells were seeded and transfected with siRNA targeting RNMT, or non-targeting control siRNA, for 40-48 hr and then incubated with media containing [35S]-labelled Met/Cys. The [35S]-labelled signal was detected using a phosphorimager and was quantified using AIDA image analyser. For each biological replicate, samples were labelled at duplicate. Bar chart depicts the normalised mean CPM per µg of protein. SD for three independent biological replicates is indicated. Statistical significance was assessed using ANOVA followed by Dunnett'smultiple comparison test using GraphPad Prism 5.0. ns $P > 0.05$, no significant difference compared to IMEC. The sensitivity of breast cancer cells to RNMT depletion is indicated.

4.2.5 The rate of proliferation does not correlate with cellular sensitivity to RNMT depletion

Previous publications have shown that RNMT expression is important for efficient cell proliferation (Aregger and Cowling, 2013, Gonatopoulos-Pournatzis et al., 2011). Therefore, I investigated whether there was a correlation between proliferation rates and cellular sensitivity to RNMT depletion. To test this hypothesis, I compared the proliferation rate of the different cell lines in the panel. Cell number was established every 24 hr for a 96 hr period post-seeding and the cell doubling time was calculated using the growth curve. As shown in Figure 4.8, the doubling time of the cell lines ranged from 23 hr to 33 hr. It was observed that three breast cancer cell lines (HCC-1806, JIMT-1, T47D) have faster proliferation rates in comparison to IMECs (Figure 4.8). Whereas five breast cancer cell lines (MCF7, CAMA-1, BT-549, MDA-MB-231, ZR.75.1) have similar proliferation rates in comparison to IMECs. Interestingly, the fastest proliferating cells (HCC-1806, JIMT-1, T47D) are “sensitive” to RNMT depletion. However, as described in the previous chapter, the breast cancer cell line MCF7, which was found to be the cell line most dependent on RNMT for survival (Figure 3.3 and Figure 3.4), displayed the slowest proliferation rate of the cell lines tested. Therefore, from the small number of cell lines investigated here, there is no apparent correlation between proliferation rates and cellular sensitivity to RNMT depletion.

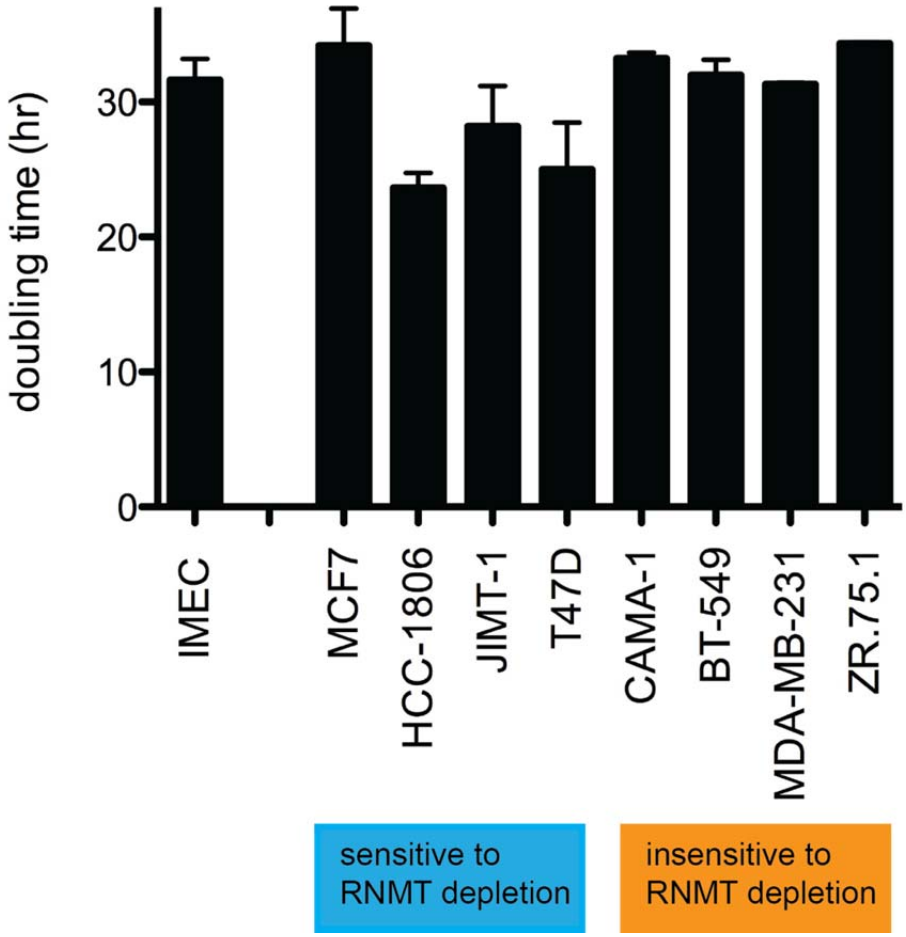


Figure 4.8 Proliferation rate of IMECs and breast cancer cells does not correlate with cellular sensitivity to RNMT depletion

Cells were plated and counted every 24 hr for 96 hr using a cell counter. For each independent biological replicate, the cells were counted in triplicate and the mean cell doubling time established. Bar chart depicts the mean cell doubling time for each cell line and mean SD for two independent biological replicates. The sensitivity of breast cancer cells to RNMT depletion is indicated.

4.2.6 c-Myc protein expression does not correlate with cellular sensitivity to RNMT depletion

The c-Myc onco-protein is deregulated in an estimated 70% of human cancers, and despite significant efforts, there are currently no therapeutic strategies to inhibit Myc function in cancer (Dang, 2012). A study from our lab demonstrated that inhibition of cap methylation selectively kills cells with elevated c-Myc expression (Fernandez-Sanchez et al., 2009a). Taken together these observations raise the possibility that breast cancer cells with elevated c-Myc expression may exhibit an enhanced dependency on RNMT for survival. To investigate whether there was a relationship between c-Myc expression and cellular sensitivity to RNMT depletion in the breast cancer cell panel, I determined c-Myc protein levels across the cell panel using immunoblot analysis with an anti-c-Myc antibody. Previous experiments have shown that the anti-c-Myc antibody is able to specifically detect c-Myc protein in immunoblot experiments (data not shown). In agreement with previous observations in our laboratory, IMECs have undetectable levels of c-Myc (Figure 4.8). The immunoblot experiment also revealed that c-Myc is differentially expressed across the breast cancer panel (Figure 4.9). It was found that breast cancer cells have either high (HCC-1806, JIMT-1, T47D and CAMA-1), intermediate (BT-549 and ZR.75.1), or low (MCF7 and MDA-MB-231) c-Myc protein expression. However, no correlation between c-Myc protein expression and cellular sensitivity to RNMT depletion could be observed.

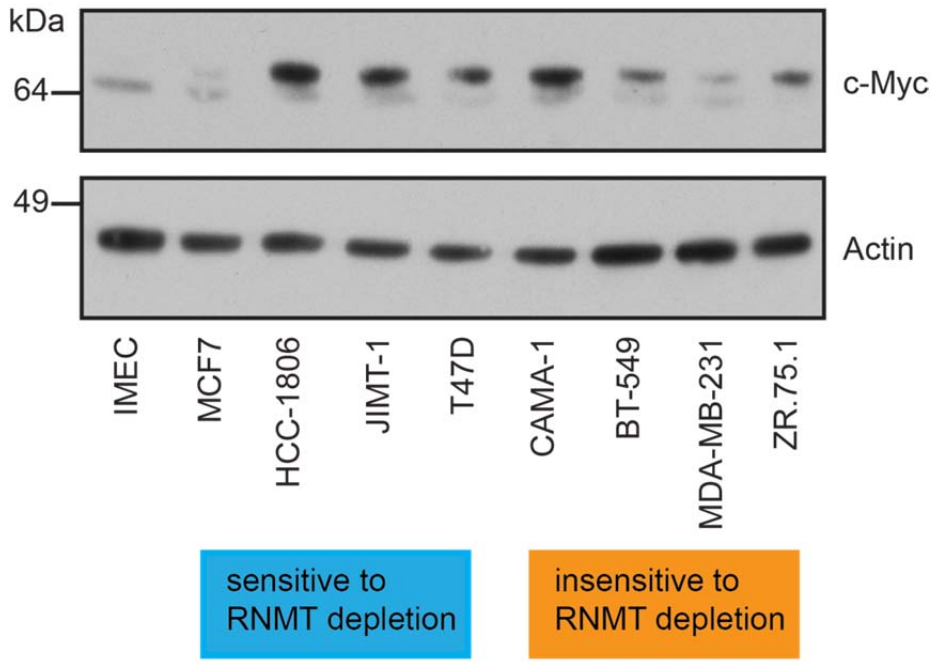


Figure 4.9 c-Myc protein expression in IMECs and breast cancer cell panel does not correlate with cellular sensitivity to RNMT depletion

Protein extracts were analysed by immunoblotting with the indicated antibodies. Actin serves as loading control. Result is representative of two independent biological replicates. The sensitivity of breast cancer cells to RNMT depletion is indicated.

4.3 Discussion

The results presented in this chapter demonstrated that cellular sensitivity to RNMT depletion does not correlate with cellular RNMT expression, RNMT enzymatic activity, global protein synthesis levels, basal cell proliferation rate and c-Myc protein expression.

4.3.1 RNMT protein expression

RNMT was not found to be expressed to higher levels in our breast cancer cell line panel, compared to immortalised mammary epithelial cells (IMECs). Moreover, RNMT was not observed to be differentially expressed between breast cancer cells “sensitive” and those “insensitive” to RNMT depletion. These results suggest that the enhanced dependency of breast cancer cells on RNMT for survival is not due to elevated RNMT expression. Consistent with my findings, a search of the ONCOMINE (a web based microarray database) revealed that RNMT mRNA expression is not higher in breast tumour tissue, in comparison to non-cancerous tissue. However, since experimental overexpression of RNMT has previously been shown to transform mammary epithelial cells, it is possible that elevated RNMT expression could contribute to breast cancer (Cowling, 2009a). In future, it could be important to examine RNMT protein expression in patient derived breast tumours, and to explore whether a correlation exists between RNMT expression in breast cancer and patient prognosis.

4.3.2 RNMT enzymatic activity

RNMT catalyses the methylation of the guanosine cap and cellular mRNA cap methyltransferase levels are dependent on RNMT expression (Cowling, 2009b). In this chapter it was revealed that the RNMT activity (cap methyltransferase) levels in the cell line panel do not correlate with cellular sensitivity to RNMT depletion. Moreover, it was observed that breast cancer cells generally have lower RNMT activity levels in comparison to IMECs. Surprisingly, this suggests that RNMT activity levels are lower in breast cancer cells, in comparison to non-transformed cells. However, it is important to note that the cap methyltransferase assay uses a single piece of *in vitro* transcribed RNA to measure cap methylation, and this may not accurately reflect the *in vivo* situation. Moreover, although demethylation of the mRNA methyl cap has not previously been described, it is possible that a cap demethylase exists in human cells and this would likely influence the cellular cap methyltransferase levels independent of RNMT activity.

The fact that cap methyltransferase levels are equivalently reduced in the cell line panel suggests that siRNA-mediated RNMT depletion is comparable in these cell lines. Despite efficient depletion of RNMT expression in all the cell lines, only a two-fold decrease in cellular cap methyltransferase activity was detected. There are several possibilities which may explain why significant cap methyltransferase levels remain in RNMT depleted cells. Firstly, it is possible that RNMT activity is upregulated in response to RNMT depletion. Secondly, it is possible that the cap methyltransferase activity that remains results from another, as yet unidentified, cellular cap methyltransferase. However, since RNMT depletion results in a two-fold decrease in cap methyltransferase levels,

there is no other cellular cap methyltransferase that can fully compensate for the loss of RNMT activity/ function.

4.3.3 Cellular proliferation rate

Cellular proliferation rate was found not to correlate with cellular sensitivity to RNMT depletion. Several breast cancer cell lines were shown to have similar proliferation rates in comparison to IMECs. This indicates that, at least in culture conditions, breast cancer cells do not always proliferate faster than non-cancerous cells. However, breast cancer cells may have a selective advantage *in vivo* which confers a faster proliferation rate.

4.3.4 Protein synthesis rate

The enhanced proliferation of cancer cells is often dependent on the deregulation of mRNA translation (Blagden and Willis, 2011, Johnson et al., 1976) and the translation of most mRNAs are dependent on the methyl cap structure (Furuichi and Shatkin, 2000). This chapter demonstrated that cells with heightened protein synthesis levels do not display an enhanced dependency on RNMT for survival. It has been reported the methyl cap is rate-limiting for the translation of a subset of mRNAs. These mRNAs are termed as “methyl cap sensitive” mRNAs (Cowling, 2009a, Cole and Cowling, 2009). It remains unknown whether a correlation exists between the translation of “methyl cap sensitive” mRNAs and cellular sensitivity to RNMT depletion, and this is an issue that should be explored further.

It has been previously been shown that methyl capped RNA is translated more efficiently compared to capped RNA (Gillian-Daniel et al., 1998, Drummond et al., 1985). It remains a possibility that the differential cellular dependency on RNMT for survival is due to a differential ability to translate capped or methyl capped RNA. I did not explore this issue due to time restrictions, but in the future it would be of interest to compare the rate of translation of capped and methyl capped RNA in the cell line panel.

The data presented in this chapter demonstrated that cellular sensitivity to RNMT depletion is not due to a differential reduction in global protein synthesis. Consistent with my findings, Cowling *et al* reported that RNMT depletion does not significantly impact global protein synthesis (Cowling, 2009a). Detectable levels of RNMT expression remain following siRNA transfection and it is possible that the low levels of RNMT that remain may be enough to maintain efficient protein synthesis. Thus, it cannot be concluded from this data that RNMT expression is not required for efficient protein synthesis. Moreover, it is possible that another enzyme exists which is functionally redundant with RNMT and is able to support protein synthesis by promoting mRNA cap methylation in response to RNMT depletion. To explore this issue further, the levels of mRNA cap methylation on endogenous transcripts should be measured by performing anti-methyl cap antibody immunoprecipitations on cellular RNA followed by real-time PCR.

Furthermore, it is important to note that the 35S technique used to measure global protein synthesis is not sensitive enough to detect a reduction in the translation of a small subset of mRNAs. Thus, the differential dependency of

cells on RNMT for survival could be due to a differential reduction in the translation of a small subset of mRNAs. This issue should be explored in the future.

4.3.5 eIF4E, the mRNA cap binding protein

The eIF4E-methyl cap interaction is essential for mRNA translation. Studies have shown that eIF4E depletion has limited effects on global protein synthesis, while specifically reducing the translation of “eIF4E-dependent” mRNAs (De Benedetti and Graff, 2004, Graff et al., 2007, Rinker-Schaeffer et al., 1992). These mRNAs generally encode for proteins that are known regulators of cell growth and survival, including oncogenic proteins (Mamane et al., 2004, De Benedetti and Graff, 2004, Koromilas et al., 1992, Rosenwald et al., 1993, Kevil et al., 1995). Since eIF4E binding to the methyl cap is essential for eIF4E-mediated mRNA translation, it is possible that a reduction in cellular cap methylation (RNMT expression) will specifically reduce the translation of oncogenic “eIF4E-dependent mRNAs” (Sonenberg et al., 1979, Sonenberg et al., 1980). The differential response of breast cancer cells to RNMT depletion may be related to a differential dependency on “eIF4E-dependent” proteins for cell survival. eIF4E is a c-Myc-transcriptional target gene and it has previously been shown that c-Myc increases the expression of eIF4E (Jones et al., 1996, Schmidt, 2004). Moreover, it has previously been reported that Myc upregulates the cap methylation of the eIF4E transcript (Cole and Cowling, 2009). Thus, it is likely that eIF4E protein expression is sensitive to changes in the levels of cellular mRNA cap methylation (RNMT expression levels). It would therefore be of interest to explore whether there is

a relationship between eIF4E expression and RNMT dependency in breast cancer cells.

4.3.6 c-Myc protein expression

Expression of the oncoprotein c-Myc is frequently deregulated in human cancers and no therapeutic strategies currently exists to target c-Myc function in cancer (Dang, 2012). It has previously been shown that c-Myc upregulates mRNA cap methylation and this is essential for c-Myc-driven protein synthesis, cell transformation and proliferation (Fernandez-Sanchez et al., 2009a, Cole and Cowling, 2009). Moreover, it has been reported that the inhibition of cap methylation selectively kills cells with elevated c-Myc expression (Fernandez-Sanchez et al., 2009a). However, the data in this chapter demonstrated that there was no correlation between c-Myc protein expression and cellular sensitivity to RNMT depletion. This suggests that the differential sensitivity of breast cancer cells to RNMT depletion is not due to differences in c-Myc expression. It must be noted that c-Myc expression in the breast cancer cell lines panel does not correlate with the rate of proliferation and therefore c-Myc is not likely to be a driving force in the associated tumours.

4.3.7 Summary

In summary, the data presented in this chapter did not reveal any cellular parameters that determine cellular sensitivity to RNMT depletion. These results suggest that RNMT depletion only affects the translation of a subset of proteins since the global translation rates remains unchanged. It remains

unknown whether particular genetic mutations in breast cancer cells confer sensitivity to RNMT depletion, and this issue is explored in the next chapter.

Chapter 5: Investigation of the relationship between the PI3K pathway and RNMT

5.1 Introduction

In the third chapter I demonstrated that RNMT depletion impaired the proliferation of a subset of breast cancer cells, but did not significantly affect the proliferation of immortalised mammary epithelial cells (IMECs). The fact that a subset of breast cancer cells are more dependent on RNMT for survival, in comparison to non-transformed cells, suggests that therapeutically targeting RNMT could be an effective anti-cancer strategy for a subset of breast tumours.

The efficacy of a targeted cancer therapy is often dependent on selecting the most receptive patient population. Therefore, in an attempt to understand why only certain breast cancer cells are sensitive to RNMT depletion, I explored whether basic cellular parameters correlated with cellular sensitivity to RNMT depletion. The results presented in the fourth chapter, found no relationship between RNMT expression, RNMT activity, protein synthesis rate, basal proliferation rate or cellular sensitivity to RNMT depletion.

This current chapter focuses on exploring the genetic determinants of sensitivity to RNMT depletion. Specifically, I investigate whether the presence of an activating mutation in the gene phosphoinositide 3 kinase catalytic alpha (PIK3CA), which encodes the p110α catalytic subunit of PI3K, sensitises cells to RNMT depletion. Moreover, I explore whether activation of the PI3K pathway correlates with cellular sensitivity to RNMT depletion.

5.1.1 The PI3K pathway

Protein kinases are key regulatory enzymes that can change the properties of a substrate by attaching a phosphate group to serine, threonine, or tyrosine resides. Phosphoinositide 3-kinases (PI3Ks) are a family of heterodimeric lipid kinases that, upon stimulation, catalyse the production of the second messenger phosphatidylinositol 3,4,5- trisphosphate ($PI(3,4,5)P_3$), which in turn activates downstream kinases, such as 3-phosphoinositide dependent kinase 1 (PDK1) and AKT. The three different classes of PI3K are classified according to their structure and substrate specificity. Only class I PI3Ks are able to produce ($PI(3,4,5)P_3$) from the substrate phosphatidylinositol 4,5 bisphosphate ($PI(4,5)P_2$) (Vanhaesebroeck et al., 2012, Vanhaesebroeck et al., 2010, Fruman and Rommel, 2014). Since only class I PI3Ks have been implicated in cancer pathogenesis this chapter introduction focuses on class I PI3Ks only.

Class I PI3Ks are receptor regulated kinases that consist of a regulatory subunit and catalytic subunit. Four distinct isoforms of the catalytic subunit (p110α, p110β, p110γ and p110δ) have been identified in mammals and each of these are able to combine with different regulatory subunits (Vanhaesebroeck et al., 2010, Zhao and Vogt, 2008b, Vanhaesebroeck et al., 2012). The different catalytic and regulatory subunits of mammalian class I PI3Ks are shown in Figure 5.1.

Although the four p110 isoforms have identical catalytic activities, they have distinct and non-redundant biological functions. p110α and p110β expression is ubiquitous but p110γ and p110δ expression is predominately leukocyte-specific. Studies have shown that knockout of p110α or p110β in mice is embryonic lethal, whereas knockout of p110γ or p110δ in mice is non-lethal but results in

defective immune responses (Vanhaesebroeck et al., 2012, Vanhaesebroeck et al., 2010, Zhao and Vogt, 2008b). There is increasing evidence that p110α contributes to human cancer development and progression. For example, activating mutations in PIK3CA, the gene encoding the p110α catalytic subunit, are frequently found in cancer and several studies have shown these mutations to be oncogenic (Samuels et al., 2004a, Isakoff et al., 2005, Ikenoue et al., 2005, Bader et al., 2006, Kang et al., 2005). Although the genes encoding the non-α p110 isoforms are not found to be mutated in cancer, the wild-type non-α p110 proteins are oncogenic when overexpressed in cells (Zhao and Vogt, 2008b). Activating mutations in PIK3CA will be discussed further in section 5.1.3.

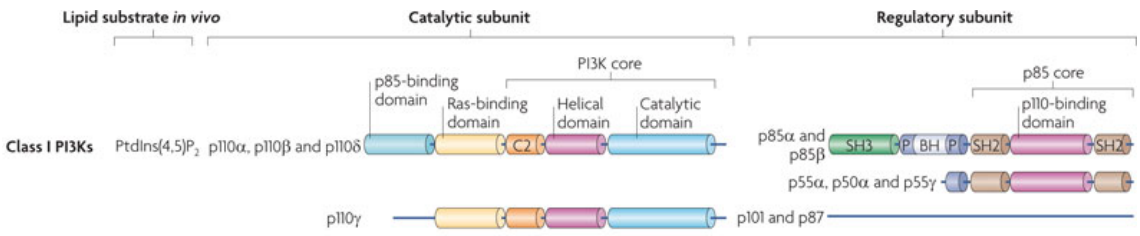


Figure 5.1 The catalytic and regulatory subunits of mammalian class I PI3Ks

Class I PI3Ks consist of a regulatory subunit and a catalytic subunit. In mammals, four isoforms (p110α, p110β, p110δ and p110γ) of the p110 catalytic subunit have been identified. The catalytic subunits p110α, p110β and p110δ associate with the regulatory subunits p50α, p55α, p55γ, p85α and p85β. The p110γ subunit associates with the regulatory subunits p101 and p87. All Class I PI3Ks catalytic subunits consist of a PI3K core subunit that consists of a C2 domain, a helical domain and a catalytic domain. The p110α, p110β and p110δ subunits also contain a p85-binding domain and a Ras-binding domain. The p110γ subunit contains a Ras-binding domain. All p85, p55 and p50 regulatory subunits have two SH2 domains and a p110 binding domain. The p101 and p87 have no identifiable domains and lack homology to other known proteins. The functions of the individual p85 regulatory subunits are unknown. The functions of the individual catalytic subunits are described in the text. Adapted from (Vanhaesebroeck et al., 2010).

5.1.2 Canonical PI3K signalling

PI3K signalling regulates key cellular processes such as cell proliferation, growth and survival. A summary of PI3K-Akt signalling is shown in Figure 5.2. The binding of ligands to receptor tyrosine kinases (RTKs), such as human epidermal growth factor (HER2), initiates a cascade of events that ultimately lead to PI3K activation. Ligand binding to the receptor results in receptor dimerization and auto-phosphorylation of tyrosine residues, followed by the recruitment of src homology 2 (SH2) domain containing proteins to the plasma membrane–bound receptor. PI3K is one such protein that is recruited. The SH2-domain of the p85 subunit of PI3K physically associates with the activated RTKs via the phospho-tyrosine residues (Liu et al., 2009a, Zhao and Vogt,

2008b). Alternatively, p85 can be recruited via an interaction with adaptor proteins, such as insulin receptor substrate 1, which interacts with the RTKs (Sun et al., 1991). Furthermore, PI3K can also associate with the plasma membrane via activated G protein coupled receptors (Stephens et al., 1994). The recruitment of PI3K to the activated receptor abolishes the suppressive effect of p85 on the catalytic activity of p110α, as well as enabling PI3K to co-localise with its plasma membrane-bound substrate, $PI(4,5)P_2$ (Yu et al., 1998). Furthermore, an association between p110α, the catalytic subunit of PI3K, and the small G-protein Ras enhances PI3K activation (Rodriguez-Viciana et al., 1994). Activated PI3K is then able to phosphorylate $PI(4,5)P_2$ to produce $PI(3,4,5)P_3$. The catalytic activity of PI3K is antagonised by the tumour suppressor phosphatase and tensin homolog (PTEN), which dephosphorylates $PI(3,4,5)P_3$ to produce $PI(4,5)P_2$ (Myers et al., 1998, Maehama and Dixon, 1998). The plasma membrane-bound $PI(3,4,5)P_3$ is then able to recruit proteins, such as the serine-threonine kinase Akt (James et al., 1996, Burgering and Coffer, 1995) and its activating kinase PDK1 (Alessi et al., 1997a), to the plasma membrane via an interaction with their pleckstrin homology (PH)-containing domains. Upon recruitment of Akt to $PI(3,4,5)P_3$, a conformational change occurs in Akt which enables PDK1 to phosphorylate Akt at Thr 308 (Alessi et al., 1997b, Milburn et al., 2003). Although phosphorylation of Akt at Thr 308 leads to a substantial increase in Akt activity, maximal Akt activity requires further phosphorylation of Akt at Ser 473, which is mediated by mTORC2 (Sarbassov et al., 2005). Following phosphorylation and activation, Akt translocates from the membrane to the nucleus where it is able to phosphorylate a diverse range of substrates (Manning and Cantley, 2007). Through the activation of these substrates, Akt is able to regulate a host of

cellular processes such as transcription, protein synthesis and cell growth (Manning and Cantley, 2007, Hers et al., 2011). For example, Akt regulates protein synthesis by signalling through the TSC complex, Rheb and mTORC1 to the downstream effectors 4E-BP and S6K1 (Laplante and Sabatini, 2012). In addition, Akt promotes cell proliferation by phosphorylating several proteins involved in the regulation of the cell cycle (Manning and Cantley, 2007). For example, phosphorylation of p27(Kip1) by Akt prevents p27(Kip1)-mediated cell cycle arrest, which results in sustained cell proliferation (Liang et al., 2002, Viglietto et al., 2002).

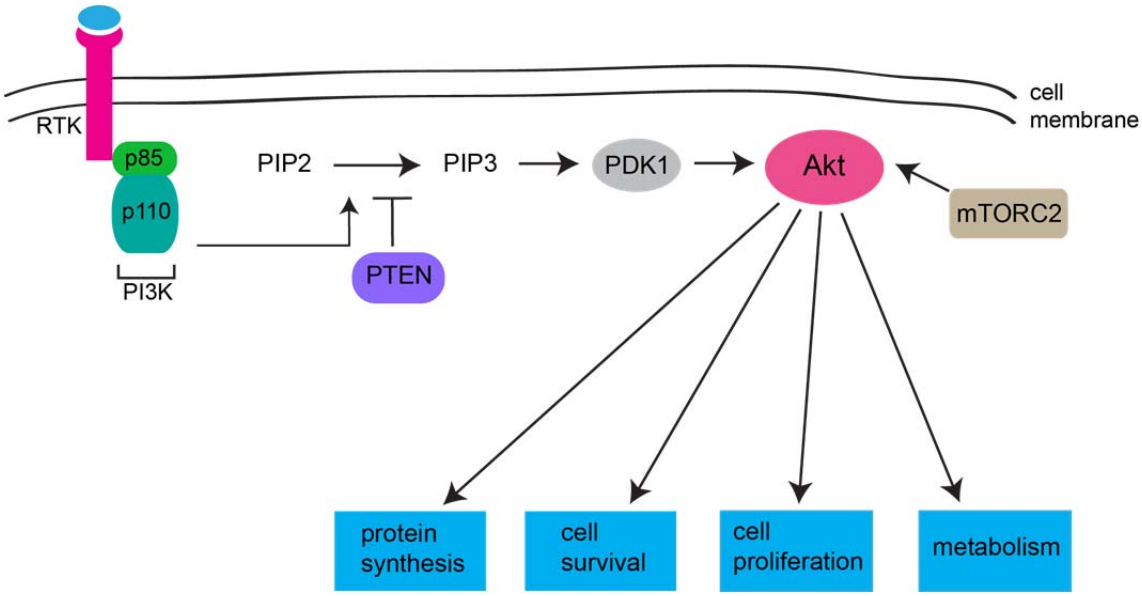


Figure 5.2 An overview of PI3K-Akt signalling

Schematic illustrates a simplified version of PI3K-Akt signalling. Ligand (such as growth factor or hormone) binding activates RTKs, which in turn activates PI3K. PI3K consists of a p85 regulatory subunit and a p110 catalytic subunit. PI3K phosphorylates the membrane bound $PI(4,5)P_2$ and produces $PI(3,4,5)P_3$. This reaction is reversed by PTEN. $PI(3,4,5)P_3$ stimulates the activation of Akt by PDK1 and mTORC2. PDK1 phosphorylates Akt on Thr 308, mTORC2 phosphorylates Akt on Ser 473 . Activated Akt phosphorylates several downstream targets which lead to the activation of several of cellular processes such as cell growth, cell survival, cell proliferation and metabolism. PI3K signalling is described in detail in the text.

5.1.3 Activating mutations in PIK3CA

As mentioned above, class I PI3Ks are heterodimers consisting of a p85 regulatory and a p110 catalytic subunit. PIK3CA is the gene coding for the p110α catalytic subunit of PI3K. Genetic cancer studies have demonstrated that PIK3CA mutations frequently occur in most human cancers (Samuels and Waldman, 2010, Samuels et al., 2004b) and PIK3CA somatic mutations are found in an estimated 18-40% of breast tumours (Bachman et al., 2004, Campbell et al., 2004, Lee et al., 2005, Levine et al., 2005, Saal et al., 2005, Network, 2012, Banerji et al., 2012, Stephens et al., 2012). The variation in the

reported frequency of PIK3CA mutations most likely reflects experimental differences across the studies; such as differences in the patient populations, tissue preparation and sample size. It remains controversial whether PIK3CA mutations in breast cancer patients correlate with clinical prognosis. Some studies have shown that breast cancer patients with PIK3CA mutations have a poor prognosis (Lai et al., 2008, Mangone et al., 2012, Barbareschi et al., 2007), whereas other have reported an improved prognosis in patients with PIK3CA mutations (Kalinsky et al., 2009, Barbareschi et al., 2007, Dumont et al., 2012).

Somatic mutations in PIK3CA are single nucleotide substitutions, and 80% of the PIK3CA mutations in human cancers are found in one of three “hot spot” mutations (E542K, E545K, H1047R), however, numerous rare cancer associated mutations have also been reported (Zhao and Vogt, 2008b). Exogenous overexpression of the hotspot p110α mutant proteins in various cell lines has shown that these mutations are oncogenic (Bader et al., 2006, Ikenoue et al., 2005, Isakoff et al., 2005, Zhao et al., 2005). Moreover, exogenous overexpression of most of the rare cancer-associated mutant p110α proteins is also sufficient to induce cell transformation (Gymnopoulos et al., 2007). In addition, several transgenic mice studies have shown that expression of the p110α hot spot mutants (E545K and H1047R) result in mammary tumour formation, but the precise mechanisms of PIK3CA-induced mammary tumorigenesis have not yet been established (Adams et al., 2011, Meyer et al., 2013, Liu et al., 2011, Koren and Bentires-Alj, 2013). A significant number of studies have shown that exogenous expression of PIK3CA activating mutants result in increased PI3K lipid kinase activity and constitutive Akt activation (Ikenoue et al., 2005, Kang et al., 2005, Isakoff et al., 2005, Gymnopoulos et al.,

2007). Data from several independent laboratories have suggested that mutations in different regions of p110α have different mechanisms by which they increase PI3K activity (Huang et al., 2008, Gymnopoulos et al., 2007, Miled et al., 2007, Zhao and Vogt, 2008a). Furthermore, a transgenic mouse study showed that hotspot mutations in either the helical or kinase domain of p110α lead to mammary tumour formation (Meyer et al., 2013).

Although, it is well established that exogenous expression of oncogenic PIK3CA results in constitutive Akt activation (Ikenoue et al., 2005, Gymnopoulos et al., 2007, Isakoff et al., 2005), a correlation between endogenous activating PIK3CA mutations and Akt activation in breast cancer has not been established. For example, two studies found no relationship between PIK3CA activating mutations and enhanced Akt signalling in breast cancer cells (Dan et al., 2010, Stemke-Hale et al., 2008). Despite minimal Akt activation in breast cancer cells with oncogenic PIK3CA mutations, evidence suggests that activating mutants of p110α contributes to the survival of these cells. For example, the presence of an activating PIK3CA mutation can sensitise breast cancer cells to certain inhibitors of the PI3K pathway (Serra et al., 2008b, Sanchez et al., 2011, Weigelt et al., 2011a, Lehmann et al., 2011, Brachmann et al., 2009a, O'Brien et al., 2010, Tanaka et al., 2011, Brachmann et al., 2009b). Moreover, a study from the Bardelli laboratory showed that the knockin of activating mutations (E545K or H1047R) into wild-type PI3KCA in human immortalised breast epithelial cells, as well as non-cancerous MCF10A breast cells, results in cell transformation and can sensitise cells to everolimus, an mTORC1 inhibitor (Di Nicolantonio et al., 2010, Di Nicolantonio et al., 2008).

5.1.4 Balance of PTEN and PI3K signalling in the cell

Since PI3K and PTEN have opposing catalytic activities, it is tempting to equate a gain of PI3K function, to a PTEN loss of function. However, the fact that PIK3CA and PTEN mutations have been found to co-occur in breast cancer, indicates that these mutations may have different (or at least additive) functional effects in breast cancer (Stemke-Hale et al., 2008, Perez-Tenorio et al., 2007). Furthermore, it has been reported that a gain of PI3K induces cellular changes that are different from those conferred by PTEN loss of function. For example, several studies have shown that PTEN loss correlates with enhanced Akt activation in a range of human cancer cells, but no relationship exists between PIK3CA activating mutations and Akt activation (Dan et al., 2010, Stemke-Hale et al., 2008, Vasudevan et al., 2009b). Interestingly, a study showed that breast cancer cells with oncogenic mutations in PIK3CA, which also exhibit minimal Akt activation, have a reduced dependency on Akt signalling for tumorigenicity. These findings suggest that PIK3CA mutations may contribute to oncogenesis at least partly via Akt-independent mechanisms. For example, breast cancers cells with activating PIK3CA mutations can display an enhanced dependence on PDK1 and serum and glucocorticoid regulated protein kinase 3 (SGK3) for survival. (Vasudevan et al., 2009b). It is important to note that the catalytic antagonism of PTEN and PI3K is not the only determinant of the balance between cellular PTEN and PI3K signalling. It is likely that the different cellular localisation of PTEN and PI3K, as well as their interactions with different proteins, could influence the balance between PI3K and PTEN signalling in the cell (Zhao and Vogt, 2008b).

The fact that breast cancer cells with oncogenic PIK3CA can display different drug sensitises from those with loss of PTEN function, provides strong evidence

that a gain of PI3K and loss of PTEN activities contribute to cancer pathogenesis differently. A large body evidence from preclinical and clinical studies shows that gain of PIK3CA function correlates with PI3K pathway inhibitor response in breast cancer (Serra et al., 2008b, Sanchez et al., 2011, Weigelt and Downward, 2012, Weigelt et al., 2011b, Lehmann et al., 2011, Brachmann et al., 2009b, O'Brien et al., 2010, Tanaka et al., 2011, Janku et al., 2011). However, whilst loss of PTEN is known to be a robust activator of PI3K signalling (Stemke-Hale et al., 2008), the relationship between PTEN loss and PI3K pathway inhibitor response in breast cancer is not as clear (Sanchez et al., 2011, Weigelt et al., 2011a, Lehmann et al., 2011, Tanaka et al., 2011, Brachmann et al., 2009a, O'Brien et al., 2010, Weigelt and Downward, 2012). It is important to note that one study demonstrated that the mutational status of the PI3K pathway did not predict the sensitivity of a panel of 39 cancer cell lines to 25 inhibitors of the PI3K pathway (Dan et al., 2010). Cancers are highly heterogeneous and it likely that the response to inhibitors of different components of the PI3K pathway may be limited to those tumours whose survival is dependent the targeted oncogenic protein.

5.1.4 Hyper-activation of PI3K signalling in breast cancer

The PI3K pathway is frequently mutated in a wide range of human cancers resulting in hyper-activation of PI3K signalling (Wood et al., 2007, Liu et al., 2009a, Thomas et al., 2007) and a number of studies have demonstrated a role for PI3K hyper-activation in cancer development and progression (Chang et al., 1997, Vogt et al., 2010, Sugimoto et al., 1984, Bader et al., 2006, Gymnopoulos et al., 2007, Kang et al., 2006). As discussed in section 1.9.4, the PI3K-Akt-

mTOR pathway regulates mRNA translation. It has been shown that the deregulation of mRNA translation by hyper-activated PI3K signalling is essential for PI3K-mediated oncogenesis (Hsieh et al., 2010, Skeen et al., 2006). The PI3K pathway is the most frequently mutated pathway in breast cancer and an estimated 60% of breast tumours display hyper-activated PI3K signalling (Miller et al., 2011, Polyak and Metzger Filho, 2012, Network, 2012, Wood et al., 2007). The most commonly observed mechanisms of PI3K signalling hyper-activation found in breast cancer include: loss of PTEN or inositol polyphosphate-4-phosphatase function; activating mutation, or overexpression, of the p110α catalytic subunit of PI3K, Akt or Ras; inactivating mutation of p85α and the amplification of RTKs such as HER2 and fibroblast growth factor receptor (Miller et al., 2011). It is important to note that activating mutations in the gene PIK3CA, which encodes for the p110α catalytic subunit of PI3K, is one the most frequently mutated genes found in breast cancer (Wood et al., 2007, Banerji et al., 2012, Stephens et al., 2012, Polyak and Metzger Filho, 2012, Network, 2012).

5.1.5 Therapeutic targeting of the PI3K pathway

Since the PI3K pathway is frequency mutated in human cancers and is a key regulator of processes essential for cancer growth it is not surprising that components of the PI3K pathway are viewed as attractive therapeutic targets in cancer. In recent years there have been significant efforts to develop specific inhibitors against several components of the PI3K pathway for use in anti-cancer therapy and several inhibitors of this pathway are currently in clinical trials (Weigelt and Downward, 2012, Liu et al., 2009a, Rodon et al., 2013).

Emerging data from clinical studies show that inhibitors of the PI3K pathway have limited anti-cancer effects when used as single agents, and the emergence of drug resistance is frequent. In an attempt to improve the efficacy of therapeutic PI3K inhibitors, there is significant effort to identify combination therapies of PI3K inhibitors with other anti-cancer therapies and to establish the genetic determinants of PI3K pathway inhibitor response (Fruman and Rommel, 2014).

5.2 Results

5.2.1 PI3K pathway mutations found in the breast cancer panel

Previous results have shown that four breast cancer cells lines are sensitive to RNMT depletion (MCF7, HCC-1806, JIMT-1 and T47D), whereas four breast cancer cells are insensitive to RNMT depletion (CAMA-1, BT-549, MDA-MB-231 and ZR.75.1) (Figure 3.3 and Figure 3.4). However, the mechanism of cellular sensitivity to RNMT depletion remains undetermined. In an attempt to identify genetic mutations that may confer cellular sensitivity to RNMT depletion, the Catalog of Somatic Mutations In Cancer (COSMIC) database was used to identify the cancer-associated somatic mutations found in the breast cancer cell panel (Forbes et al., 2010) (Figure 5.2). It was found that three out of the four breast cancer cell lines which are sensitive to RNMT depletion (MCF7, T47D, JIMT-1) have activating mutations (E545K, H1047R and C420R, respectively) in the gene PIK3CA, which encodes the p110α catalytic subunit of PI3K. These mutations in PIK3CA result in single amino acid substitutions. Studies have shown that exogenous expression of these p110α mutants results in PI3K activation and oncogenesis (Bader et al., 2006, Ikenoue et al., 2005, Isakoff et al., 2005). E545K and H1047R are hot spot mutations, whereas C420R is a rare cancer associated mutation (Zhao and Vogt, 2008b). In addition, HCC-1806 cells, which are also sensitive to RNMT depletion, have an LKB1 deletion. Loss of LKB1 function has previously been shown to result in enhanced mTORC1 activity (Shaw et al., 2004). Moreover, it was found that three out of four the breast cancer cell lines (CAMA-1, BT-549 and ZR.75.1) which are insensitive to RNMT depletion have mutations that lead to loss of PTEN function. As discussed in section 5.1.4, both loss of PTEN and gain of PIK3CA are commonly found in breast tumours and result in hyper-activation of PI3K

signalling (Miller et al., 2011). Overall these findings show that the breast cancer cells which are sensitive to RNMT depletion have either a PIK3CA activating mutation, or an LKB1 deletion.

Cell line	Gene	Mutation (amino acid)	References
MCF7	FLT3 PIK3CA	C368Y E545K	
HCC-1806	CDKN2A CDKN2 a (p14) KDM6A LKB1 TP53	deletion deletion deletion deletion T256> frameshift	
T47D	P53 PIK3CA	L194F H1047R	
JIMT-1	HER-2 TP53 PIK3CA	amplification R248W C420R	Tanner., 2004
CAMA-1	NTRK1 TP53 PTEN	S475C R280T D92H	
BT-549	PTEN TP53	W274L R2495	
MDA-MB-231	ATM BRAF EGFR KRAS TP53	N10051 G464V L469W G13D R280K	
ZR.75.1	PTEN	L108R	Hollestelle., 2007; Weigelt., 2007

Figure 5.3 Known gene mutations in the breast cancer cell lines studied in this thesis

Mutational information was obtained from the online Catalogue Of Somatic Mutations In Cancer (COSMIC) database (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/) or the references indicated. Only genes that are defined by COSMIC as being cancer associated have been listed here.

5.2.2 PI3K-Akt-mTOR pathway activation in the cell panel

Mutations in the PI3K pathway that lead to deregulated PI3K signalling can render breast cancer cells sensitive to certain therapeutic inhibitors of the PI3K pathway (Weigelt and Downward, 2012). Since the breast cancer cells studied in this project were found to harbour mutations that have the potential to deregulate PI3K-Akt-mTOR signalling (Figure 5.3), an obvious question was whether PI3K pathway activation correlated with cellular sensitivity to RNMT depletion. To assess PI3K-Akt-mTOR pathway activation in the panel of breast cancer cells, and immortalised mammary epithelial cells (IMECs), the levels of phosphorylated forms of Akt (Ser 473 and Thr 308), S6K (Thr 389) and 4E-BP1 (Thr 37/46 and Thr 70) were assessed using immunoblot analysis (Figure 5.3). The activation of Akt was determined by assessing Akt phosphorylation levels at Ser 473 and Thr 308. This revealed that all the breast cancer cell lines "insensitive" to RNMT depletion (CAMA-1, BT-549, MDA-MB-231 and ZR.75.1), one of the breast cancer cell lines "sensitive" to RNMT depletion (JIMT-1) and IMECs all displayed enhanced levels of Akt phosphorylation (Ser 473). In contrast, three out the four breast cancer cell lines "sensitive" to RNMT depletion had undetectable levels of Ser 473 Akt phosphorylation. This result indicates that breast cancer cells which are sensitive to RNMT inhibition generally have lower Akt phosphorylation levels at Ser 473. In contrast, it was found that cells displayed either high (BT-549 and IMECs), intermediate (JIMT-1, CAMA-1 and ZR.75.1), or low (MCF7, HCC-1806, T47D and MDA-MB-231) levels of Thr 308 Akt phosphorylation. Strikingly, the levels of Thr 308 Akt phosphorylation across the cell panel did not appear to correlate with cellular sensitivity to RNMT depletion (Figure 5.4).

The activation of mTORC1 was assessed by monitoring the phosphorylation levels of S6K and 4E-BP1. Similar levels of S6 protein and S6 phosphorylation (Thr 389) was detected in all the cell lines, except for MCF7, which displayed substantially higher levels of S6 protein expression and S6K phosphorylation. This result is consistent with previously observations that S6K protein expression is upregulated in MCF7 cells due to gene amplification (Bärlund et al., 2000). In addition, similar levels of 4E-BP phosphorylation at Thr 37/46 and Thr 70 were detected in all the cell lines, apart from BT-549 which had increased phosphorylation at both Thr 37/46 and Thr 70. Protein expression levels of 4E-BP were variable across all the cell lines tested. Overall, there did not appear to be any correlation between cellular sensitivity and mTORC1 activity (Figure 5.4).

Taken together, these results show that breast cancer cells which are sensitive to RNMT inhibition generally display lower levels of phosphorylation of Akt at Ser 473. However, there seems to be no correlation between cellular sensitivity to RNMT depletion and global Akt phosphorylation at Ser 308, or with mTORC1 activity.

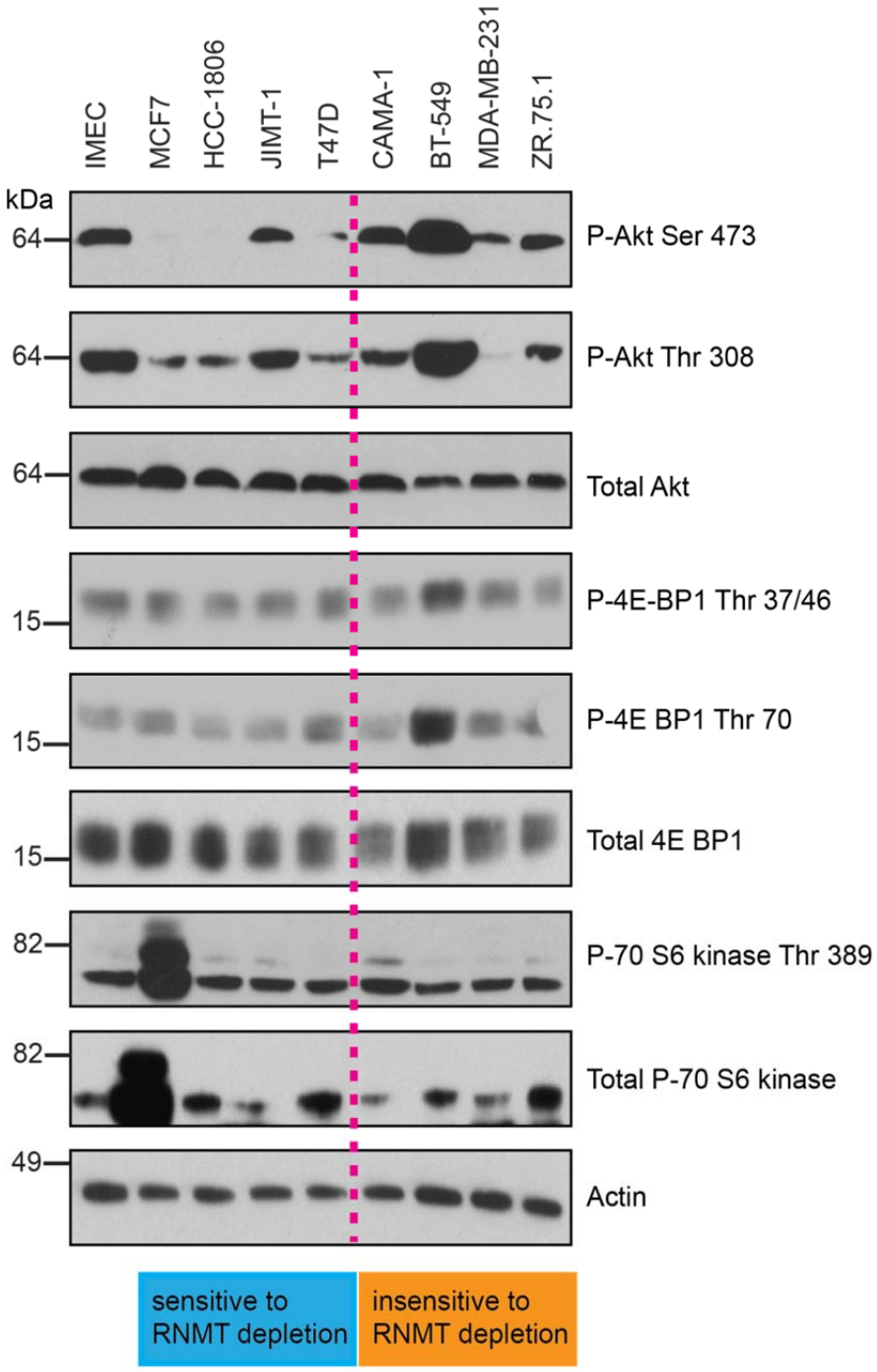


Figure 5.4 Immunoblot analysis of PI3K-Akt-mTOR pathway activation in IMECs and breast cancer cells

Protein lysates were analysed by immunoblotting with the indicated antibodies. Actin serves as loading control. Results are representative of two independent biological replicates. Sensitivity to RNMT depletion is indicated.

5.2.3 Expression of PI3K p110α activating mutants increase PI3K signalling in ZR.75.1 cells

As discussed above, the gene PIK3CA is frequently mutated in breast tumours (Network, 2012, Polyak and Metzger Filho, 2012). These PIK3CA mutants are oncogenic and the presence of activating PIK3CA mutations can sensitise breast cancer cells to certain inhibitors of the PI3K pathway (Serra et al., 2008a, Sanchez et al., 2011, O'Brien et al., 2010, Weigelt et al., 2011b, Brachmann et al., 2009a, Tanaka et al., 2011). It was observed that three out the four breast cancer cell lines (MCF7**,** HCC-1806 and JIMT-1) which are "sensitive" to RNMT depletion have activating mutations in PIK3CA (E545K, H1047R and C420R, respectively) (Figure 5.3), whereas none of the breast cancer cell lines which are "insensitive" to RNMT depletion have oncogenic mutations in PIK3CA. This raises the possibility that activating mutations in PIK3CA may contribute to cellular sensitivity to RNMT depletion. To test this, I investigated whether exogenous expression of p110α activating mutants in an "RNMT depletion insensitive" cell line can render the cell line "RNMT depletion sensitive". Previous experiments showed ZR.75.1 breast cancer cells to be insensitive to RNMT depletion (Figure 3.3 and Figure 3.4). Thus, I generated ZR.75.1 cells stably expressing Myc-tagged wild-type, or oncogenic activating mutants (E545K, H1047R, C420R), of p110α, or vector control using retroviral transduction. I was unable to detect expression of the exogenous p110α proteins using immunoblotting alone (data not shown). Therefore, to assess expression of the exogenous constructs, cell lysates were immunoprecipitated and immunoblotted using an anti-9E10 antibody that detects the Myc tag. This revealed that p110α wild-type, and activating mutants (C420R, E545K, H1047R) were expressed to similar levels (Figure 5.4A). Moreover, RNMT

expression levels were unaffected by exogenous expression of wild-type, or activating mutants, of p110α (Figure 5.5A).

Activation of Akt is essential for PI3K signalling and Akt activation has been shown to be dependent on the Akt phosphorylation at the sites Ser 473 and Thr 308 (Alessi et al., 1997b, Milburn et al., 2003, Sarbassov et al., 2005). To determine the effects of the p110α activating mutants on PI3K signalling, I assessed the levels of Akt phosphorylation by immunoblotting protein lysates with antibodies that detect Akt protein and the phosphorylated forms of Akt (Thr 308 and Ser 473). Despite similar expression levels of Akt protein, cells exogenously expressing the p110α mutants (C420R, E545K, H1047R) displayed increased Akt phosphorylation at both Ser 473 and Thr 308, compared with cells expressing wild-type p110 (Figure 5.5B). Overall these results show that although wild-type, and the three activating mutants, of p110α are expressed to similar levels (Figure 5.5A), the mutants are more effective at activating PI3K signalling (Figure 5.5B).

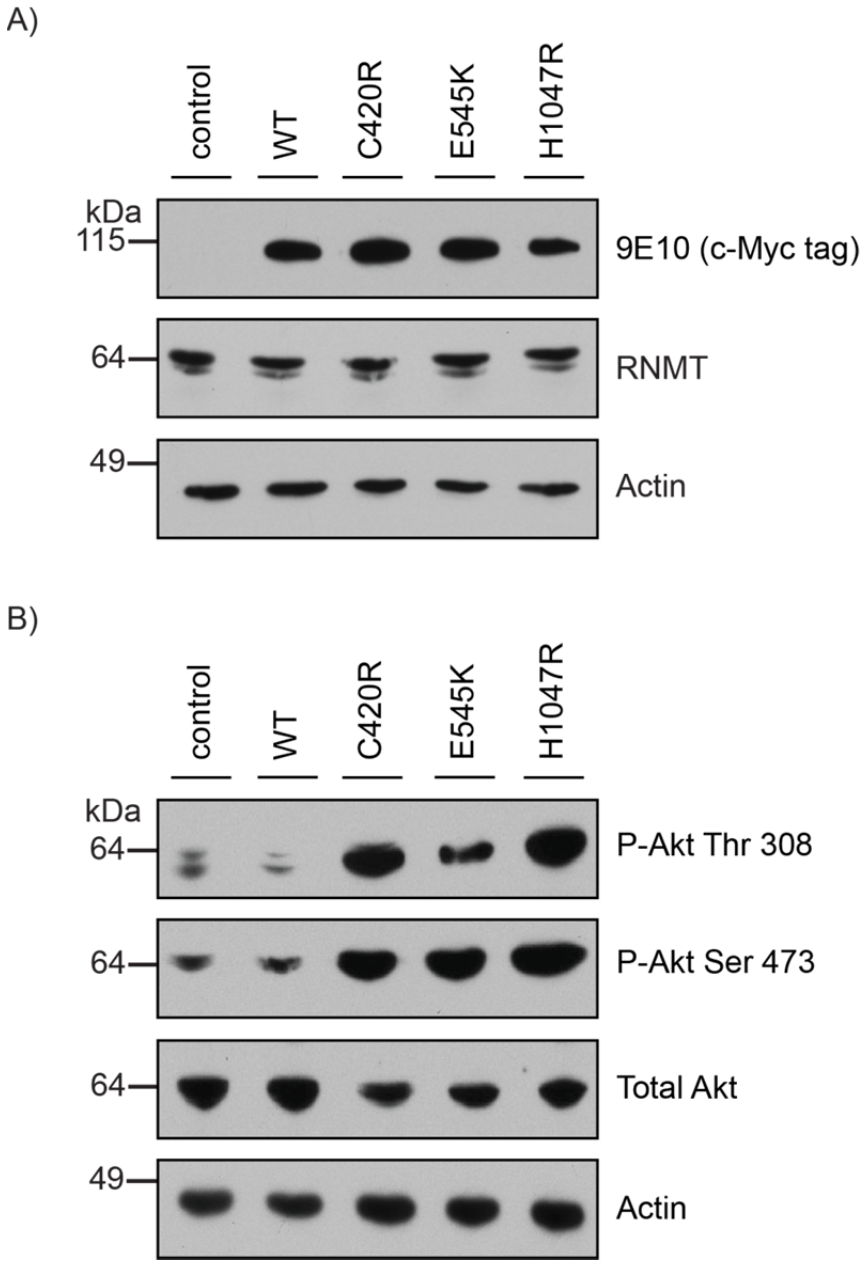


Figure 5.5 Expression of PI3K p110α activating mutants increase Akt phosphorylation in ZR.75.1 cells

Cells stably expressing Myc-tagged wild-type (WT), activating mutants (C420R, E545K, H1047R) of PI3K p110α, or vector control were lysed. (**A**) 1.5 mg of protein lysates were immunoprecipitated with anti-9E10 antibody and immunoblot analysis performed on immunoprecipitates using anti-c-Myc (9E10) antibody and on IP inputs using the anti-Actin and anti-RNMT antibodies. Actin serves as loading control. Result is representative of two independent biological experiments. (**B**) Protein lysates were analysed by immunoblotting with the indicated antibodies. Actin serves as loading control. Result is representative of two independent biological replicates.

5.2.4 Expression of PI3K p110α mutants sensitise ZR.75.1 cells to RNMT depletion

I next sought to establish whether exogenous expression of the activating p110α mutant proteins can render ZR.75.1 cells sensitive to RNMT depletion. It was first important to assess whether exogenous expression of oncogenic p110α mutant proteins (C420R, E545K and H1047R) affected RNMT depletion levels. Therefore, cells were transfected with siRNA targeting RNMT, or a non-targeting siRNA, for 72 hr and protein lysates were then immunoblotted with antibodies against RNMT, Akt and the phosphorylated form of Akt (Ser 473). This revealed that RNMT protein was equivalently depleted in cells expressing wild-type, or the activating mutants, of p110α (Figure 5.6). This result was also produced using an independent siRNA targeting RNMT (siRNA RNMT 3) (data not shown). Consistent with the previous result (Figure 5.5B), there was a substantial increase in Akt phosphorylation (Ser 473) in cells expressing mutant p110α ,compared to cells expressing wild-type p110α (Figure 5.6). In addition, RNMT depletion resulted in reduced Akt phosphorylation (Ser 473) levels in cells expressing vector control, p110α wild-type and the p110α C420R mutant. To determine whether exogenous expression of activating p110α mutants can render ZR.75.1 cells sensitive to RNMT depletion, I assessed whether RNMT depletion preferentially inhibited the growth of cells expressing p110α activating mutants, compared to cells expressing wild-type p110α. Cells were transfected with siRNA targeting RNMT, or a non-targeting siRNA, and were counted on three consecutive days. Consistent with previous observations (Figure 3.3 and Figure 3.4), RNMT depletion does not affect the proliferation of ZR.75.1 cells (Figure 5.7A). Strikingly, it was revealed that RNMT depletion resulted in a greater decrease in proliferation in cells expressing the p110α mutants,

compared to cells expressing p110 wild-type, or vector control. Exogenous expression of each of the three oncogenic p110α mutants (C420R, E545K and H1047R) resulted in a similar reduction in proliferation in response to RNMT depletion. It is important to note that there was no difference in the basal proliferation rate of ZR.75.1 cells expressing wild-type, or the p110α mutants (data not shown).

To further expand on the above findings, cells were transfected with an siRNA targeting RNMT, or a non-targeting siRNA, and cells were counted 72 hr post-siRNA transfection. As shown in Figure 5.7B, RNMT depletion resulted in a significant decrease in proliferation in cells expressing mutant p110α, compared to cells expressing wild-type p110α, or vector control ($P \leq 0.01$, ANOVA followed by Dunnett's multiple comparison test). As expected, a similar result was produced when RNMT was depleted using an additional siRNA targeting RNMT (Figure 5.7C) ($P \leq 0.001$, ANOVA followed by Dunnett's multiple comparison test).

Overall these results demonstrate that exogenous expression of the p110α mutants (E545K, C420R and H1047R) in ZR.75.1 cells result in increased PI3K signalling and leads to an enhanced dependence on RNMT for proliferation. These results support the notion that breast cancer cells harbouring activating mutations in the gene PIK3CA have enhanced sensitivity towards RNMT depletion, which suggests that PIK3CA mutations and enhanced PI3K signalling contribute to cellular sensitivity to RNMT depletion.

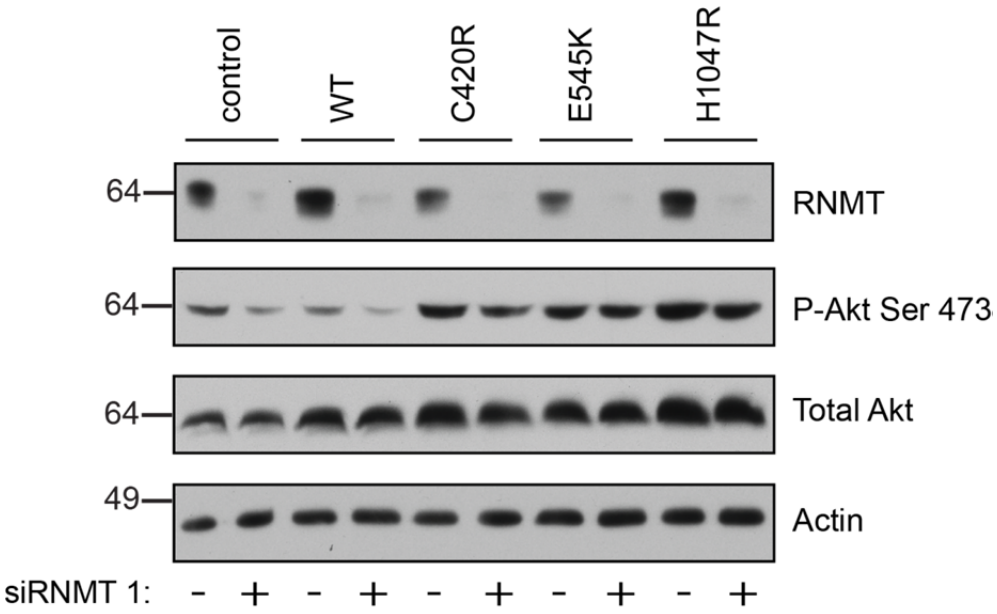
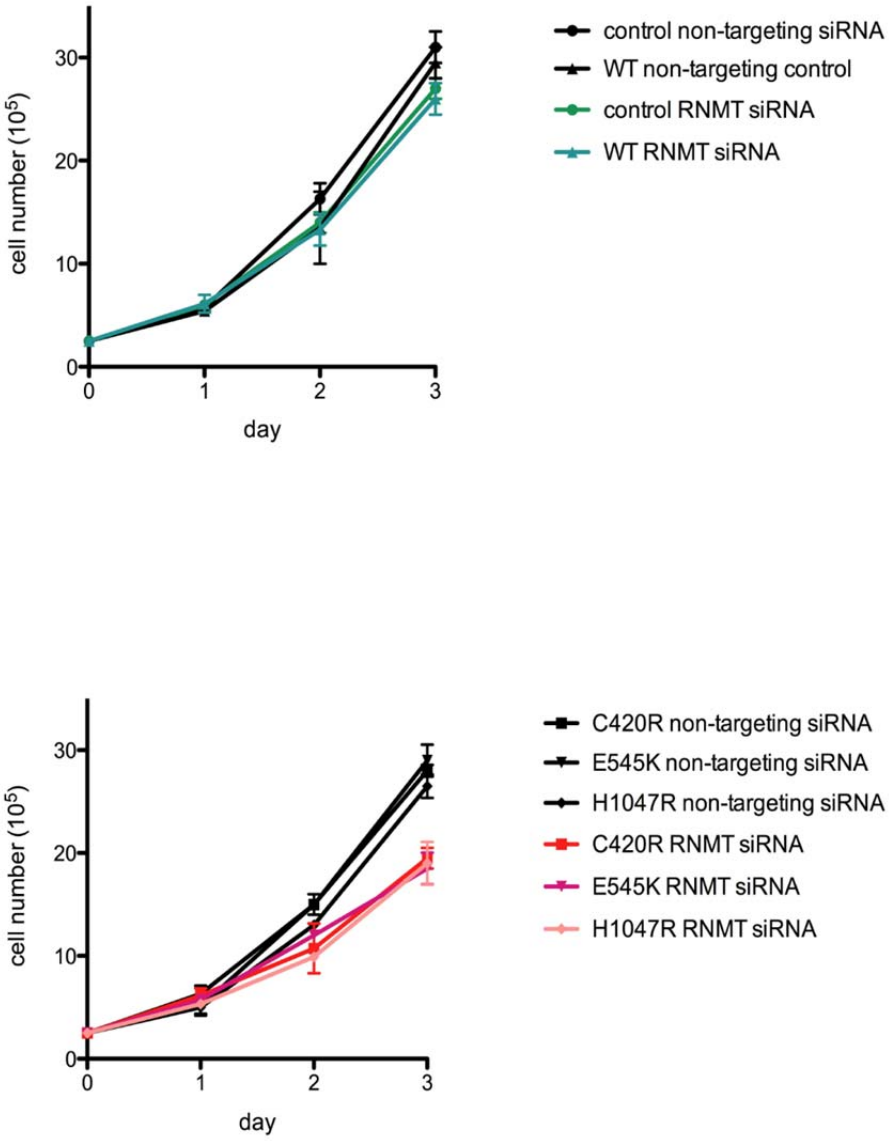


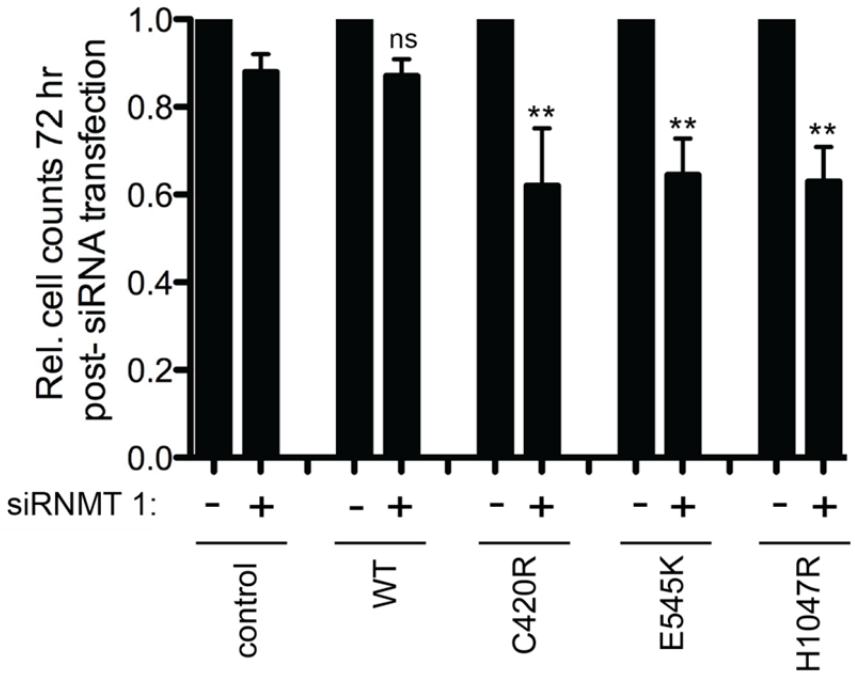
Figure 5.6 RNMT is equivalently depleted using siRNA in ZR.75.1 cells expressing either wild-type, or activating mutants, of PI3K p110α

Cells stably expressing wild-type (WT), activating mutants (C420R, E545K, H1047R) of PI3K p110, or vector control (control), were transfected with siRNA targeting RNMT (siRNA RNMT 1) (+), or non-targeting siRNA (-), for 72 hr and then lysed. Protein lysates were analysed using immunoblotting with the indicated antibodies. Actin serves as loading control.

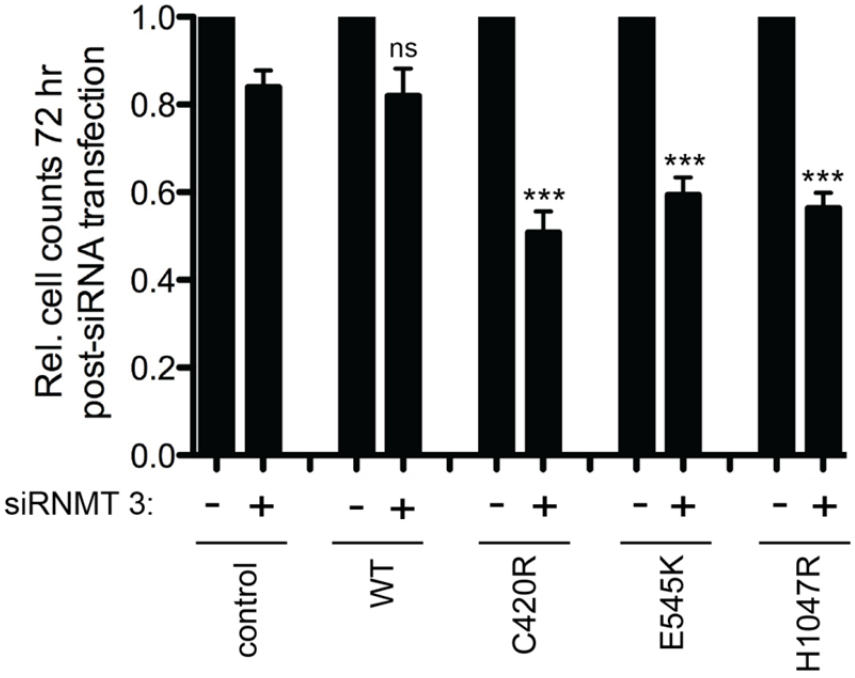
A)


cell number (10^5)
30
20
10
0
0
1
2
3
day
control non-targeting siRNA
WT non-targeting control
control RNMT siRNA
WT RNMT siRNA
C420R non-targeting siRNA
E545K non-targeting siRNA
H1047R non-targeting siRNA
C420R RNMT siRNA
E545K RNMT siRNA
H1047R RNMT siRNA

B)


Rel. cell counts 72 hr post- siRNA transfection
1.0
0.8
0.6
0.4
0.2
0.0
ns
**
**
**
siRNMT 1:
- + - + - + - + - +
control
WT
C420R
E545K
H1047R

C)


Rel. cell counts 72 hr post-siRNA transfection
1.0
0.8
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0.2
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ns
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siRNMT 3:
- + - + - + - + - +
control
WT
C420R
E545K
H1047R

Figure 5.7 Expression of PI3K p110α activating mutants in ZR.75.1 cells result in an enhanced dependence on RNMT for proliferation

Cells stably expressing wild-type (WT), activating mutants (C420R, E545K, H1047R) of PI3K p110α, or vector control (control), were seeded. (**A**) Cells were transfected with siRNA targeting RNMT (RNMT 3), or non-targeting siRNA, and were counted at triplicate on three consecutive days (day 1, 2, 3) using a cell counter. Graph shows mean cell number against days of growth and SD. (**B**) Cells were transfected with siRNA targeting RNMT (siRNA RNMT 1) (+), or a non-targeting siRNA (-), for 72 hr and were counted using a cell counter. Each bar chart depicts mean relative cell number and SD for three independent biological replicates. Statistical significance was assessed using ANOVA followed by Dunnett's multiple comparison test using GraphPad Prism 5.0. ns $P > 0.05$, no significant difference compared to control cells transfected with siRNA RNMT; ** $P \leq 0.01$; *** $P \leq 0.001$. (**C**) same as (**B**) except cells transfected with siRNMT 3.

5.2.5 Expression of PI3K p110α activating mutants in IMECs increase PI3K signalling

It has previously been shown that exogenous overexpression of oncogenic p110α in MCF10A non-transformed breast cells results in constitute Akt activation and leads to transformation (Isakoff et al., 2005). Furthermore, “knockin” of hot spot mutations into the gene PIK3CA (E545K and H1047R) in MCF10A cells can sensitise them to everolimus, an mTORC1 inhibitor (Di Nicolantonio et al., 2008, Di Nicolantonio et al., 2010). Previous experiments have shown that RNMT depletion does not significantly affect the proliferation of immortalised mammary epithelial cells (IMECs) (Figure 3.1 and Figure 3.4). To further explore whether activating mutations in PIK3CA can contribute to cellular sensitivity to RNMT depletion, I assessed whether exogenous expression of activating p110α mutants in IMECs can sensitise cells to RNMT depletion. To do this, I generated IMECs stably expressing Myc-tagged wild-type p110α, or activating mutants (C420R, E545K and H1047R) using retroviral transduction. I was unable to detect expression of the p110α constructs by immunoblotting alone (data not shown). To assess the expression of the exogenous constructs, cell lysates were immunoprecipitated and immunoblotted using an anti-9E10 antibody that detects the Myc tag. Despite equal IP protein input, as judged by similar actin levels, the p110α mutants E545K and H1047R were expressed to lower levels in comparison to wild-type p110α, or the C420R mutant (Figure 5.8A). Furthermore, RNMT expression levels were not shown to be affected by exogenous expression of wild-type, or oncogenic mutants, of p110α protein (Figure 5.8A). To determine the effects of activating mutants of p110α on the activation of the PI3K signalling, I assessed the levels of Akt phosphorylation (Ser 473 and Thr 308) by immunoblotting protein lysates with antibodies that

detect Akt protein and the phosphorylated forms of Akt (Ser 473 and Thr 308). It was found that cells expressing exogenous p110α mutants (C420R, H1047R, E545K) displayed increased Akt phosphorylation at both sites, compared with cell expressing wild-type p110α, or vector control (Figure 5.8B). Despite lower expression of the p110α mutants E545K and H1047R, the three p110α mutants display a similar increase in the levels of Akt phosphorylation. Overall these results show that exogenous expression of the p110α mutants (C420R, H1047R, E545K) in IMECs result in enhanced PI3K signalling.

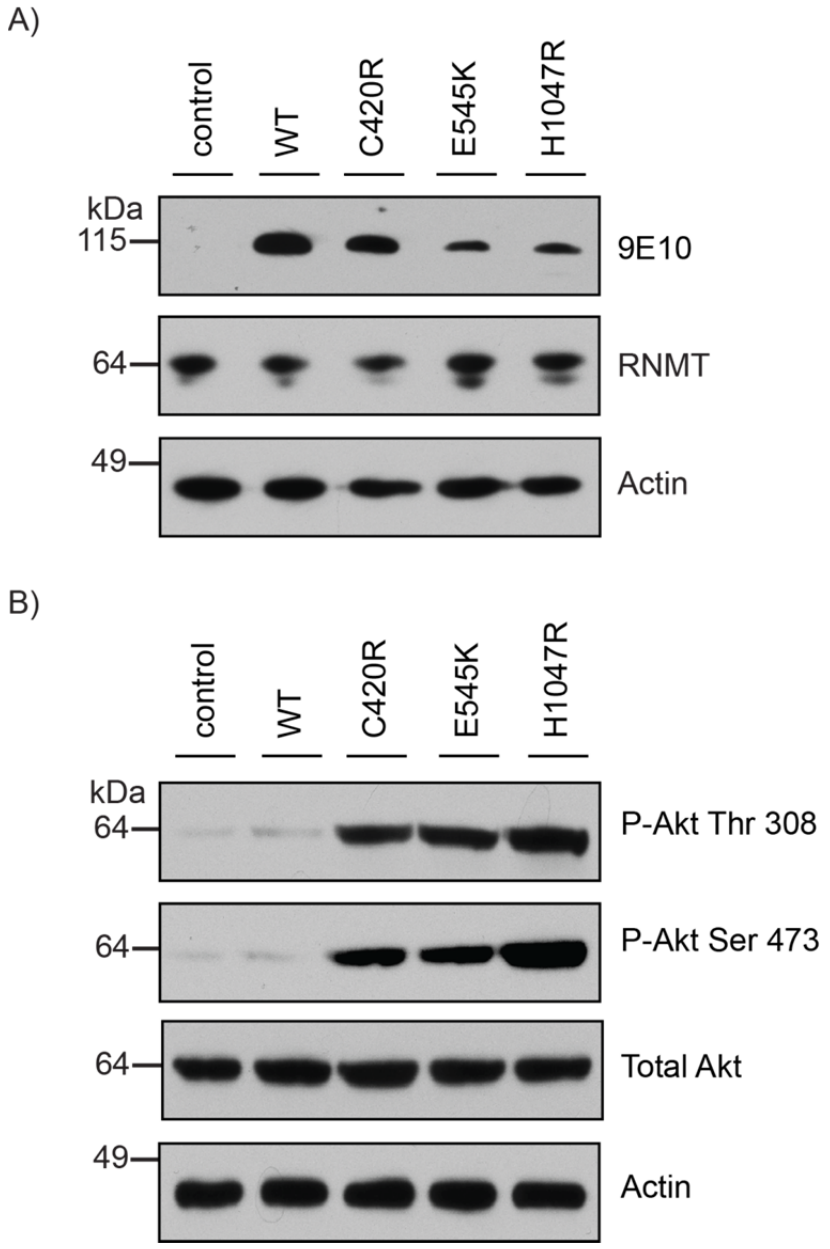

Figure 5.8 Expression of PI3K p110α activating mutants increase Akt phosphorylation in IMECs

Cells stably expressing Myc-tagged wild-type (WT), activating mutants (C420R, E545K, H1047R) of PI3K p110α, or vector control were lysed. (**A**) 1.5 mg of protein lysates were immunoprecipitated with anti-9E10 antibody and immunoblot analysis performed on immunoprecipitates using anti-c-Myc (9E10) antibody and on inputs using the anti-Actin and anti-RNMT antibodies. Actin serves as loading control. Result is representative of two independent biological replicates. (**B**) Protein lysates were analysed by immunoblotting with the indicated antibodies. Actin serves as loading control. Result is representative of two biological replicates.

5.2.6 Expression of p110α activating mutants can sensitise IMECs to RNMT depletion

I next sought to establish whether exogenous expression the activating p110α mutants (C420R, E545K and H1047R) can render IMECs sensitive to RNMT depletion. It was first important to assess whether exogenous expression of p110α in IMECs affected RNMT depletion levels. Therefore, cells were transfected with siRNA targeting RNMT, or a non-targeting siRNA, for 72 hr and protein lysates were immunoblotted with antibodies against RNMT, Akt and the phosphorylated forms of Akt (Ser 473). It was found that expression of the exogenous constructs did not affect RNMT depletion levels. Consistent with a previous result (Figure 5.8B), exogenous expression of the p110α oncogenic mutants resulted in a substantial increase in Akt phosphorylation. Moreover, RNMT depletion resulted in reduced Akt Ser473 phosphorylation levels in cells exogenously expressing the C420R and E545K p110α mutants. To assess whether RNMT depletion preferentially inhibited the growth of IMECs expressing p110α activating mutants, compared to cells expressing wild-type p110α. Cells were transfected with a siRNA targeting RNMT, or a non-targeting siRNA, and were counted 72 hr post-siRNA transfection. Consistent with previous results (Figure 3.1 and Figure 3.4), RNMT depletion did not significantly affect the proliferation of IMECs (Figure 5.10). Strikingly, RNMT depletion resulted in a significantly greater decrease in proliferation in cells expressing mutant p110α, compared to cells expressing wild-type p110α, or vector control ($P \leq 0.5$, ANOVA followed by Dunnett's multiple comparison test). It is important to note that exogenous expression of wild-type p110α, or the activating mutants, did not affect the basal proliferation rate of IMECs (data not shown).

Overall these results show that exogenous expression of p110α mutants in non-transformed mammary epithelial cells lead to hyper-activation of PI3K signalling and result in an enhanced dependence on RNMT for proliferation. These results support the notion that activating mutations in the gene PIK3CA and enhanced PI3K signalling contribute to cellular sensitivity to RNMT depletion.

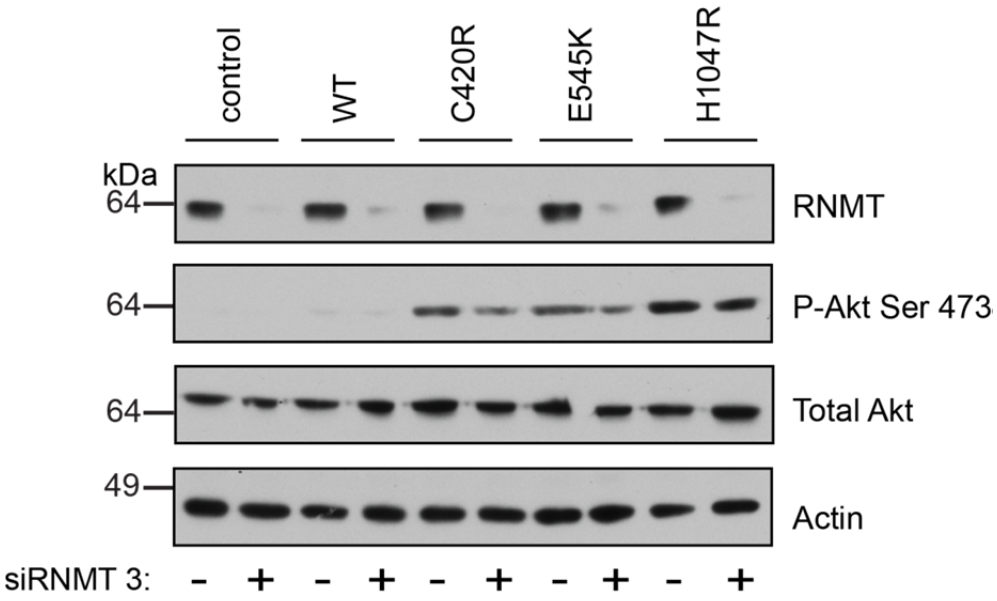

Figure 5.9 RNMT is equivalently depleted in IMEC expressing either wild-type, or activating mutants, of PI3K p110α

IMECs stably expressing wild-type (WT), activating mutants (C420R, E545K, H1047R) of PI3K p110α, or vector control (control), were seeded and transfected with siRNA targeting RNMT (siRNMT 3) (+), or non-targeting siRNA (-), for 72 hr. Protein lysates were analysed using immunoblotting with the indicated antibodies. Actin serves as loading control.

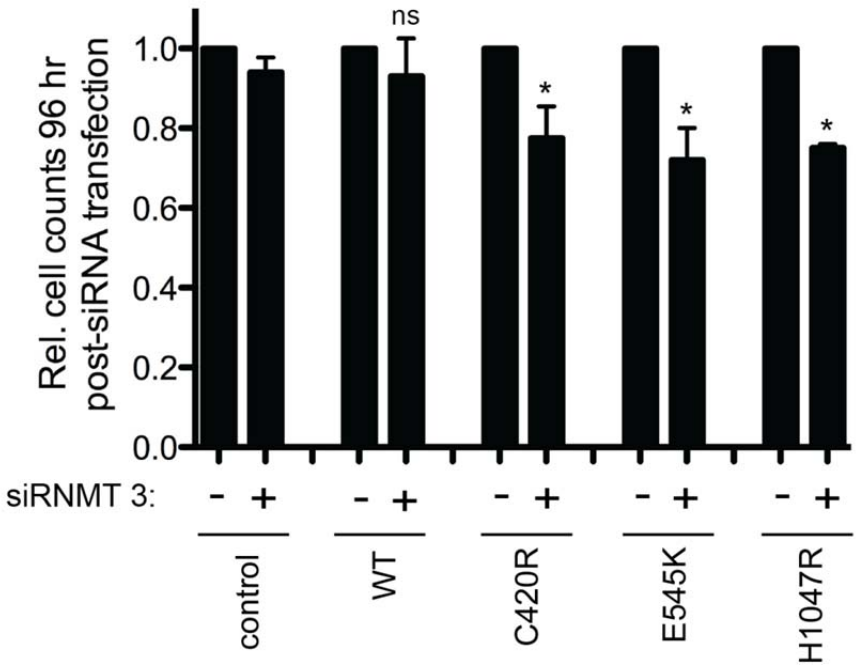

Figure 5.10 Expression of PI3K p110α activating mutants in IMECs result in an enhanced dependence on RNMT for proliferation

Cells stably expressing wild-type (WT), activating mutants (C420R, E545K, H1047R) of PI3K p110, or vector control (control), were transfected with siRNA targeting RNMT (siRNA RNMT 3) (+), or a non-targeting siRNA (-), for 72 hr and were counted using a cell counter. Each bar chart depicts mean relative cell number and SD for three independent biological replicates. Statistical significance was assessed using ANOVA followed by Dunnett's multiple comparison test using GraphPad Prism 5.0. ns $P > 0.05$, no significant difference compared to control cells transfected with siRNA RNMT; * $P \leq 0.5$.

5.2.7 Pharmacological inhibition of PI3K reverses the sensitivity of T47D cells to RNMT depletion

The results presented above demonstrated that exogenous overexpression of p110α mutants in both ZR.75.1 cells, and IMECs result in constitutive PI3K signalling (Figure 5.5 and Figure 5.8) and lead to an enhanced dependency on RNMT for proliferation (Figure 5.7 and 5.10). These results suggest that enhanced PI3K signalling may contribute to cellular sensitivity to RNMT depletion. Thus, I hypothesised that downregulation of PI3K signalling in cells which are sensitive to RNMT depletion should reverse the sensitivity. To test this hypothesis, I assessed the combined effect of RNMT depletion and pharmacological inhibition of PI3k on the proliferation of T47D cells. Previous experiments have shown T47D cells to be sensitive to RNMT depletion (Figure 3.3 and Figure 3.4). Cells were treated with 0.05 μM of GDC-0941 (Folkes et al., 2008a), a selective inhibitor of all four isoforms of class I PI3K, and were transfected with siRNA targeting RNMT, or a non-targeting siRNA. 72 post-treatment, cells were either counted, or lysed for immunoblot analysis. I decided to used 0.05 μM of GDC-0941 on the basis of findings from a study which showed that treatment of T47D cells with 0.05 μM resulted in PI3K inhibition but did not significantly affect cellular proliferation (O'Brien et al., 2010). Immunoblotting of cell lysates with antibodies against RNMT, Akt and the phosphorylated form of Akt (Ser 473) revealed that treatment of cells with GDC0941 decreased Akt phosphorylation (Ser 473) to undetectable levels. In addition, it was found that RNMT protein expression was depleted to equivalent levels in the presence of GDC-0941, or DMSO (Figure 5.11A). Consistent with previous observations (Figure 3.3 and Figure 3.4), it was revealed that RNMT depletion in T47D cells resulted in a statistically significant two-fold decrease in

cell number, compared to cells transfected with non-targeting siRNA ($P \leq 0.001$, ANOVA followed by Tukey's multiple comparison test) Strikingly, RNMT depletion in the presence of GDC-0941 did not result in a significant decrease in cell number, compared to cells transfected with non-targeting siRNA ($P > 0.05$, ANOVA followed by Tukey's multiple comparison test) Moreover, RNMT depletion in the presence of GDC-0941 resulted in a significant decrease in cell number, compared to cells treated with DMSO ($P \leq 0.01$, ANOVA followed by Tukey's multiple comparison test) (Figure 5.11B). Overall these results show that pharmacological inhibition of PI3K signalling desensitises T47D cells to RNMT depletion. Thus, the above findings support that hypothesis that enhanced PI3K signalling may contribute to the sensitivity of breast cancer cells to RNMT depletion.

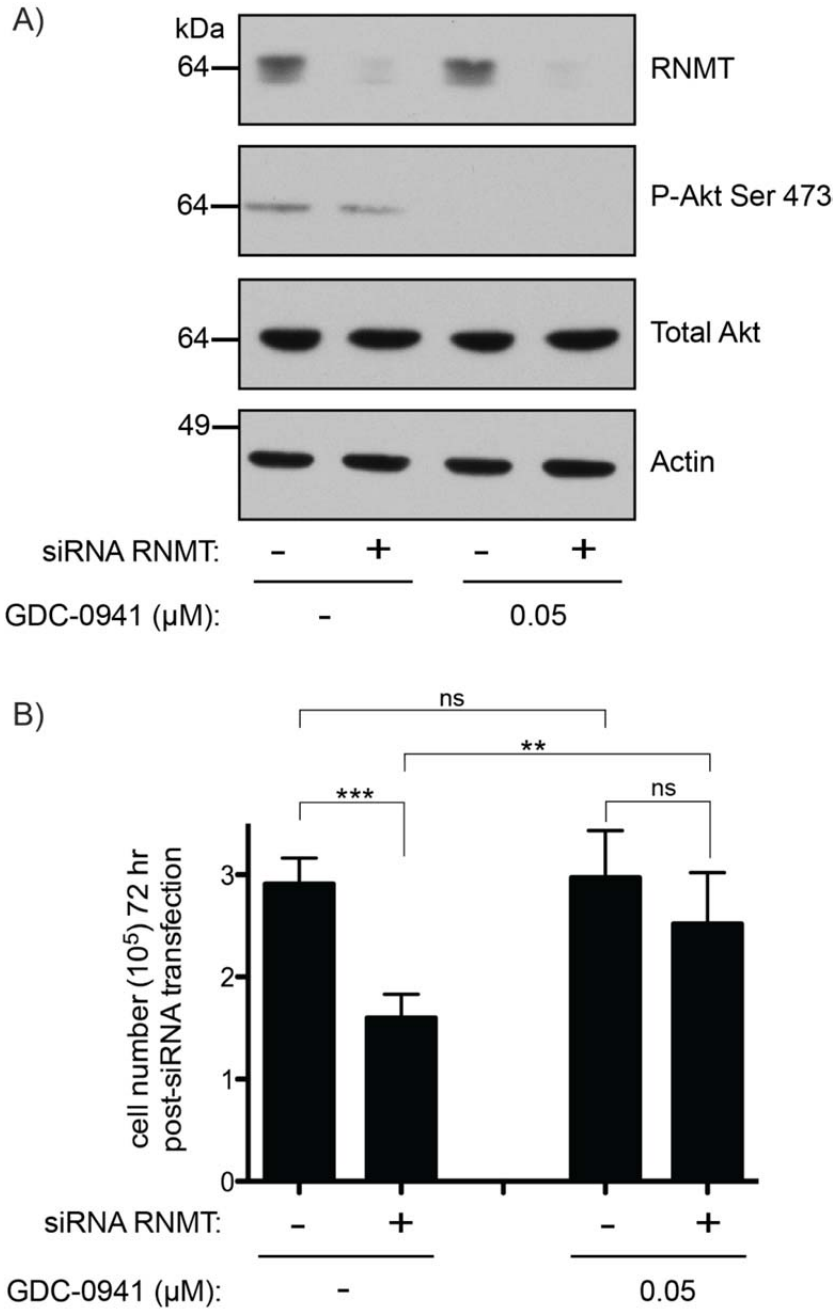

Figure 5.11 Pharmacological inhibition of PI3K reverses the RNMT-depletion induced proliferation defect of T47D cells

Cells were seeded and transfected with siRNA targeting RNMT (RNMT 3) (+), or non-targeting control siRNA (-). 4 hr post-siRNA transfection, cells were treated with 0.05 μM of GDC-0941, or equal volume of DMSO control (-). (**A**) 72 hr post-siRNA transfection, protein lysates were extracted and analysed by immunoblotting with the indicated antibodies. Actin serves as loading control. Result is representative of two independent biological replicates. (**B**) 72 hr post siRNA transfection, cells were counted at triplicate using a cell counter. Bar chart depicts mean normalised cell number and SD for three independent biological replicates. Statistical significance was assessed using ANOVA followed by Tukey's multiple comparison tests using GraphPad Prism 5.0. ns $P > 0.05$; ** $P \leq 0.01$,*** $P \leq 0.001$.

5.3 Discussion

5.3.1 PIK3CA activating mutations

Results presented in chapter 3 demonstrated that RNMT depletion impairs the proliferation of a subset of breast cancer cell lines, in comparison to non-transformed mammary epithelial cells. However, the mechanism of cellular sensitivity to RNMT depletion remained undetermined. In this chapter it was revealed that three out of the four breast cancer cell lines which are sensitive to RNMT depletion (MCF7, T47D, JIMT-1) have activating mutations (E545K, H1047R and C420R, respectively) in the gene PIK3CA, which encodes the p110α catalytic subunit of PI3K. It was demonstrated that exogenous expression of p110α activating mutants in cells increases PI3K signalling and leads to an enhanced dependence on RNMT for proliferation. This does not exclude the possibility that other genetic mutations may also contribute to RNMT dependency in breast cancer cells. To fully establish the validity of my results, it will be important to explore the effects of RNMT depletion on the proliferation of a larger breast cancer cell line panel and perform mechanistic studies.

My findings support the idea that targeting RNMT would be most suitable for breast cancer patients harbouring mutations in PIK3CA. The efficacy of a targeted cancer therapy is often dependent on selecting the most receptive patient population. Since an estimated 18-40% of breast tumours harbour mutations in PIK3CA, these findings could have important therapeutic implications. The mechanisms by which PIK3CA activating mutations enhance dependence on RNMT expression for proliferation are currently unclear. It is possible that breast cancer cells with activating mutations in PIK3CA are highly

dependent on a subset of proteins for survival and mRNA cap methylation (RNMT) is required to facilitate the translation of these cancer survival proteins. Moreover, it is also possible that the PI3K pathway influences the translation of specific proteins by directly modulating RNMT activity/ target specificity. In future, it would therefore be of interest to explore the mechanisms of RNMT dependence in breast cancer cells.

5.3.2 PI3K signalling and sensitivity to RNMT depletion

The three activating p110α mutants used (E545K, H1047R and C420R) in this study each have single amino acid substitutions in different regions of the p110α protein (helical, kinase and C2, respectively). Data from several studies has shown that mutations in different regions of p110α enhance PI3K activity by differing mechanisms (Huang et al., 2008, Gymnopoulos et al., 2007, Miled et al., 2007, Zhao and Vogt, 2008a). It was demonstrated that each of the p110α mutants were shown to enhance PI3K signalling equivalently, which is consistent with results from previous studies (Ikenoue et al., 2005, Gymnopoulos et al., 2007). Moreover, the three p110α activating mutants were equally able to enhance cellular sensitivity to RNMT depletion. These observations may indicate that enhanced PI3K signalling contributes to the

Inhibition of PI3K signalling in a breast cancer cell line which is sensitive to RNMT depletion reversed the sensitivity. This provides additional evidence to suggest that RNMT dependency in breast cancer is related to PI3K activity/ signalling. It remains unknown whether this effect is specific to PI3K activity, or is an effect of downstream PI3K pathway signalling. It would therefore be of interest to explore whether inhibitors of other components of the PI3K signalling

pathway (such as mTOR or Akt) are able to reverse the sensitivity of breast cancer cells to RNMT depletion. Furthermore, there are four isoforms of p110 (p110α, p110β, p110δ, p110γ) and it would be on interest to explore whether p110 isoform specific inhibitors can reverse the sensitivity of breast cancer cells to RNMT depletion.

The data presented in this chapter shows that RNMT depletion reduces Akt phosphorylation levels (Ser 473) in cells expressing p110α wild-type and the C420R and E545K activating mutants. However, RNMT depletion does not reduce Akt phosphorylation levels (Ser 473) in cells expressing the H1047R activating mutant. Since RNMT depletion equivalently reduces the proliferation of cells expressing each of the three p110α activating mutants (C420R, E545K and H1047R), it is likely that changes in Akt phosphorylation levels (Ser 473) are not mediating the proliferative defect induced by RNMT depletion. This may suggest that activating mutations in PIK3CA lead to an enhanced dependency on RNMT via Akt-independent mechanisms. Vasudevan *et al*, reported that a subset of breast cancer cells with PIK3CA activating mutations have reduced dependency on Akt signalling for survival, in comparison to cells with PTEN loss (Vasudevan et al., 2009a), and instead rely on other signals including PDK1 and SGK3.Thus, it is an intriguing possibility that the differential response of breast cancer cells to RNMT depletion could be due to a dependence on PDK1 and SGK3 and this should be investigated in the future.

5.3.3 Mutations in PTEN and PI3K

PI3K activating mutations and PTEN inactivating mutations in cancer cells both increase $PI(3,4,5)P_3$ levels. Therefore, it was surprising that our breast cancer cells with activating PIK3CA mutations were sensitive to RNMT depletion, whereas the cells with loss of PTEN were not. However, emerging evidence has shown that a gain of PI3K function has different biological effects to the loss of PTEN (Zhao and Vogt, 2008b, Blanco-Aparicio et al., 2007). Moreover, several studies have reported that breast cancer cells with PIK3CA activating mutations have different drug sensitises from those cells with loss of PTEN, (Serra et al., 2011, Sanchez et al., 2011, Weigelt et al., 2011a, Weigelt and Downward, 2012, Brachmann et al., 2009b, O'Brien et al., 2010, Janku et al., 2011, Lehmann et al., 2011, Tanaka et al., 2011). Several studies have reported that PTEN loss in breast cancer correlates with Akt activation, whereas PIK3CA activating mutations do not correlate (Dan et al., 2010, Stemke-Hale et al., 2008, Vasudevan et al., 2009a). These observations are consistent with my findings that Akt Ser 473 phosphorylation levels are higher in breast cancer cells with PTEN loss, in comparison to those cells with activating mutations in PIK3CA.

It has previously been observed that certain transcripts are more dependent on the methyl cap for their translation (Cowling, 2009a). Thus, it is possible that breast cancer cells with activating mutations in PIK3CA have an enhanced dependency on the translation of these transcripts for cancer survival, and this issue should be explored further. For example, it is possible that RNMT depletion in breast cancer cells may reduce the expression of PDK1 or SGK3.

5.3.4 Targeting RNMT and mTOR for combinational therapies

Several studies have shown that breast cancer cells with activating PIK3CA mutations have enhanced sensitivity to therapeutic PI3K pathway inhibitors, such as mTOR inhibitors (Weigelt et al., 2011a). However, emerging data has shown that inhibitors of the PI3K pathway have limited anti-tumour effects when used as single agents and the emergence of drug resistance in common. Upregulation of eIF4E expression has been shown to be a mechanism of resistance to mTOR inhibitors in cancer (Sun et al., 2005, Wendel et al., 2004a, Cope et al., 2013). Since eIF4E binding to the methyl cap is essential for eIF4E-mediated translation and eIF4E-mediated oncogenesis, it may be of interest to explore whether inhibition of RNMT in combination with mTOR inhibitor treatment synergises to reduce resistance in cancer. It is possible that inhibition of RNMT also synergises with other anti-cancer treatments, and this is an issue that should be explored.

5.3.5 The non-alpha P110 isoforms

The four isoforms of p110 (p110α, p110β, p110δ, p110γ) have identical enzymatic activates, but experimental evidence suggest that they have isoform-specific cellular functions (Denley et al., 2008). The gene PIK3CA, which encodes p110α, is frequently mutated in cancer and p110α is known to be oncogenic (Zhao and Vogt, 2008b). No cancer-associated mutations have been found in the non-alpha p110 isoforms, however, it has been reported that wild-type p110β, p110δ, p110γ are oncogenic when overexpressed. In contrast, wild-type p110α is not oncogenic when overexpressed. Interestingly, unlike p110α, transformation by p110β and p110γ does not involve increased PI3K

signalling (Denley et al., 2008). Since elevated expression of the non-alpha isoforms has been observed in human cancers, it is possible that these isoforms contribute to human cancer (Benistant et al., 2000, Sujobert et al., 2005, Mizoguchi et al., 2004, Zhao and Vogt, 2008b). It is possible that the increased activity/ expression of the non-alpha p110 isoforms contribute to RNMT dependence in breast cancer cells. It would therefore be of interest to explore whether the enhanced dependence of breast cancer cells on RNMT for survival is a feature specific to the p110α isoform.

5.3.6 Summary

The results presented in this chapter provide the first evidence that expression of activating p110α mutants enhance cellular sensitivity to RNMT depletion. This suggests that activating mutations in the gene PIK3CA at least partly sensitise breast cancer cells to RNMT depletion. Since an estimated 18-40% of breast tumours have activating mutations in PIK3CA, my findings could potentially have significant therapeutic implications. My work indicates that RNMT dependence in breast cancer is related to PI3K signalling/ activity. These data strongly suggest that RNMT should be explored as a therapeutic target in breast cancers with activating mutations in PIK3CA.

Chapter 6: Final Discussion and future work

6.1 Final Summary

Breast cancer is the leading cause of cancer death in women worldwide. Although significant advances have been made in the treatment of breast cancer, new therapeutic approaches are required. Protein synthesis is often found to be deregulated in cancer and there has been significant effort into developing strategies to inhibit protein synthesis for the treatment of cancer.

The 7-methylguanosine cap structure found at the 5' end of mRNA is essential for mRNA translation and it has been shown to promote several steps in gene expression. In humans, the methylation of the guanosine cap structure is catalysed by RNA guanine-7 methyltransferase (RNMT). RNMT expression is rate-limiting for efficient mRNA translation and cell proliferation (Fernandez-Sanchez et al., 2009a, Cowling, 2009a, Aregger and Cowling, 2013). Several observations from our laboratory suggest there is strong biological rationale to explore RNMT as a therapeutic target in breast cancer. However, it has been unknown whether targeting RNMT would supress the proliferation of all cells, or has selectively towards cancer cells.

The initial aim of this thesis was to investigate the impact of RNMT depletion on the proliferation of eight breast cancer cell lines and non-transformed mammary epithelial cells. These experiments revealed that RNMT depletion selectively inhibits the proliferation of a subset of breast cancer cells, in comparison to non-transformed mammary epithelial cells. Moreover, it was found that RNMT depletion induces apoptosis in a subset of breast cancer cell

lines. Overall these results show that a subset of breast cancer cell lines exhibit an enhanced dependency on RNMT (mRNA cap methylation) for survival.

The next aim of this thesis was to understand why only certain breast cancer cell lines are sensitive to RNMT depletion. Thus, I explored whether basic cellular parameters correlated with cellular sensitivity to RNMT depletion. It was demonstrated that cellular sensitivity to RNMT depletion does not correlate with cellular RNMT expression, RNMT enzymatic activity, global protein synthesis levels, basal cell proliferation rate or c-Myc protein expression.

I next investigated whether a particular genetic mutation in the breast cancer cells conferred sensitivity to RNMT depletion. It was found that three out of the four breast cancer cell lines which are sensitive to RNMT depletion have activating mutations in the gene PIK3CA, which encodes the p110α catalytic subunit of PI3K. It was demonstrated that exogenous expression of the p110α mutants in cells result in hyper-activated PI3K signalling and lead to an enhanced dependence on RNMT for proliferation. These results suggest that activating mutations in the gene PIK3CA and enhanced PI3K signalling contribute to cellular sensitivity to RNMT depletion. My findings support the idea that targeting RNMT would be most suitable for breast cancer patients harbouring activating mutations in PIK3CA. Since an estimated 18-40% of breast tumours harbour activating mutations in PIK3CA (Bachman et al., 2004, Campbell et al., 2004, Lee et al., 2005, Levine et al., 2005, Saal et al., 2005, Network, 2012, Banerji et al., 2012, Stephens et al., 2012), these findings could have important therapeutic implications. The precise mechanisms by which PIK3CA activating mutations enhance RNMT dependency in breast cancer remain unknown.

It was found that the inhibition of PI3K signalling in an RNMT depletion sensitive breast cancer cell line reverses the sensitivity. This provides additional evidence to suggest that RNMT dependency in breast cancer is related to PI3K activity/ signalling. It remains unknown whether this effect is specific to PI3K activity, or is an effect of downstream PI3K pathway signalling.

In summary, the results presented in this thesis demonstrate that a subset of breast cancer cell lines have an enhanced dependence on RNMT for survival, in comparison to immortalised mammary epithelial cells. This is the first evidence that targeting methyl cap synthesis can selectively inhibit the proliferation of cancer cells, in comparison to non-transformed cells. Moreover, I show that activating mutations in the gene PIK3CA, which encodes the p110α catalytic subunit of PI3K, contribute to cellular sensitivity to RNMT depletion. Overall, these findings strongly suggest that RNMT should be explored as a therapeutic target in breast cancer and this could potentially open up an exciting new avenue in cancer research.

6.2 Final discussion and Future Work

Taken together the data presented in this thesis indicate that RNMT should be investigated as a therapeutic target in breast cancer. Moreover, my results suggest that there is a link between the PI3K activity and RNMT dependency in breast cancer. However, there are a lot of unanswered questions and future work is required to further understanding the findings presented in this thesis.

1) Do activating mutations in PIK3CA contribute to RNMT dependency in breast cancer?

Further work is required to fully establish whether activating mutations in PIK3CA confer sensitivity to RNMT depletion in breast cancer cells. It would be interesting to study the effect of RNMT depletion on the proliferation of a larger breast cancer cell line panel. This will help further evaluate whether there is a link between activating mutations in PIK3CA and RNMT dependency in breast cancer cells, and it may also reveal additional genetic mutations that contribute to RNMT dependency. In this study I demonstrated that exogenous expression of p110α activating mutants enhances cellular sensitivity to RNMT depletion in two independent cell lines. It would be desirable to repeat these experiments in additional breast cancer cell lines. It is important to note that the above experiments were performed using exogenous expressed p110α constructs and it may be necessary to explore how endogenous PIK3CA mutations contribute to RNMT dependency in breast cancer. Isogenic cancer cell lines are created by modifying endogenous genes using homologous gene targeting and this ensures that the gene of interest is expressed to physiological levels. Isogenic cancer cell lines can be utilised to investigate how a particular genetic mutation contributes to drug sensitivities in cancer. Therefore, isogenic cancer cell lines, in which the endogenous PIK3CA gene harbouring an activating mutation has been replaced with wild-type PIK3CA, will be used to further explore whether PIK3CA mutations contribute to RNMT dependency in cancer.

It remains unknown whether the link between RNMT dependency and PIK3CA activating mutations is specific to breast cancer. Activating mutations in PIK3CA are frequently found in several types of human cancer, such as colon cancer (Samuels and Waldman, 2010, Samuels et al., 2004a). It would therefore be of

interest to explore the effect of RNMT depletion on the proliferation of other human cancer cell lines with PIK3CA mutations. These experiments may reveal that RNMT should be explored as a therapeutic target in other types of human cancer.

It is possible that other genes contribute to RNMT dependency in breast cancer and it would be of interest to perform a genome wide siRNA screen to identify candidate genes that are key modulators of RNMT dependency in breast cancer cells.

2) Is there a link between PI3K signalling and RNMT dependency in breast cancer?

My findings suggest that RNMT dependency in breast cancer is linked to PI3K activity/ signalling. In order to investigate this link further, inhibitors against different components of the PI3K pathway (such as Akt and mTORC1) will be used to dissect the role of the PI3K pathway in RNMT dependency. In addition, there are four isoforms of p110 (p110α, p110β, p110δ, p110γ) and it will be of interest to explore whether p110 isoform specific inhibitors can reverse the sensitivity of breast cancer cells to RNMT depletion. These experiments will allow me to investigate whether RNMT dependence in breast cancer is p110 isoform specific. Overall, the above experiments will help assess whether there is a relationship between the PI3K pathway and RNMT depletion which has the potential to be exploited therapeutically.

3) What are the mechanisms of RNMT dependency in breast cancer?

The mechanisms by which activating mutations in PIK3CA enhance cellular sensitivity to RNMT depletion are not yet understood and it will be important to perform mechanistic studies to investigate this.

It is possible that the PI3K pathway influences the translation of specific transcripts by modulating RNMT target specificity. Thus, RNMT depletion may affect the proteome differently in breast cancer cells “sensitive” and in those “insensitive” to RNMT depletion and this may explain the differential dependence on RNMT for survival. It will therefore be of interest to use proteomics to compare the proteome of the breast cancer cell line panel in response to RNMT depletion.

It has been previously been shown that methyl capped RNA is translated more efficiently compared to capped RNA (Gillian-Daniel et al., 1998, Drummond et al., 1985). It is possible that the differential cellular dependence on RNMT for survival is due to a differential ability to translate capped or methyl capped RNA. Therefore, it will be of interest to compare the rate of translation of capped and methyl capped RNA in the breast cancer cell line panel. Capped and methyl capped transcripts encoding luciferase will be transfected into breast cancer cells and the translational efficiency of these transcripts will be determined by measuring luciferase activity.

It has previously been observed that certain transcripts are more dependent on the methyl cap for their translation and these are termed “methyl cap sensitive” transcripts (Cowling, 2009a). It is possible that the breast cancer cells with activating mutations in PIK3CA have an enhanced dependence on the translation of these “methyl cap sensitive” transcripts for survival. Since breast

cancer cells with activating mutations in PIK3CA have previously been shown to be highly dependent on the expression of SGK3 and PDK1 for survival (Vasudevan et al., 2009a), it would be interesting to investigate SGK3 and PDK1 protein expression levels in the RNMT depleted breast cancer cell line panel. It would also be interesting to explore the activation of components of the PI3K pathway (such as Akt and mTORC1), or of other oncogenic signalling pathways, in response to RNMT depletion in the breast cancer cell line panel.

Since IRES-dependent translation is maintained during cellular conditions which repress cap-dependent translation, it is likely that the translation of IRES-containing mRNAs will be maintained in response to RNMT depletion (Stoneley and Willis, 2004, Holcik and Sonenberg, 2005). Since many proteins which are translated in an IRES dependent manner are known regulators of cell survival, certain breast cancer cell lines may exhibit an enhanced dependence on IRES-translated proteins for survival (Liwak et al., 2012, Miskimins et al., 2001, Yang et al., 2006). Thus, it is possible that breast cancer cells insensitive to RNMT depletion are highly dependent on IRES-mediated translation for survival, whereas breast cancer cells sensitive to RNMT depletion are highly dependent on cap-dependent translation for survival. However, since the full spectrum of endogenous mRNAs translated in an IRES-mediated manner remains unknown, it may be difficult to assess whether there is a correlation between IRES-dependent translation and RNMT dependency in breast cancer cells.

The tumour suppressor protein p53 supresses cell transformation by inhibiting cell growth or inducing apoptosis. P53 is the most frequently mutated protein found in breast cancer and loss of p53 plays a key role in oncogenesis (Polyak and Metzger Filho, 2012, Vogelstein et al., 2000). Since p53 can be translated in an IRES-mediated manner, it is possible that p53 expression is maintained in

response to RNMT depletion and may be a modulator of RNMT depletion response (Yang et al., 2006). Interestingly, MCF7, the breast cancer cell line most sensitive to RNMT depletion, expresses WT p53 and would therefore likely remain responsive to p53-induced apoptosis. In the future, it would be of interest to explore whether p53 contributes to RNMT depletion response in breast cancer cells.

4) Does RNMT depletion inhibit breast tumour growth *in vivo*?

Although this study has provided the first evidence that RNMT depletion impairs the proliferation of breast cancer cell lines, it will be important to explore the *in vivo* significance of my findings using a mouse model of breast cancer. Therefore, mice with inducible RNMT knockout will be bred with an established breast cancer mouse model and the resulting transgenic mice will be used to assess whether RNMT deletion suppresses breast tumour growth *in vivo*. Moreover, it will be important to assess whether pharmacological inhibition of RNMT reduces tumour growth in a breast cancer mouse model. The results from these experiments will help further assess the suitability of RNMT as a therapeutic target in breast cancer.

5) Suitability of a specific RNMT inhibitor as an anti-cancer therapeutic drug?

There are currently no specific inhibitors against RNMT activity. However, our lab is currently screening for specific RNMT inhibitors in collaboration with the Drug Discovery Unit (College of Life Sciences, University of Dundee). To

establish the validity of my findings, it would be desirable to investigate the effects of an RNMT inhibitor on our breast cancer cell line panel. This will help us to decide whether the RNMT inhibitor should be further developed as a potential anti-cancer therapeutic drug. Since therapeutic resistance to RNMT inhibitors in breast cancer could potentially occur via the upregulation of proteins which are functionally redundant with RNMT, it will be important to extensively investigate whether other cap cellular methyltransferases exists in humans. Moreover, if therapeutic resistance to RNMT inhibitors occurs it would be of interest to perform a genome wide siRNA screen to identify candidate genes that are key modulators of resistance.

8. References

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